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Full Name of Author — Nom complet de l'auteur: ALAN FRANK COOK

Date of Birth — Date de naissance April 29 1954	Country of Birth — Lieu de naissance CANADA

Permanent Address — Résidence fixe
10236 - 71 Street, Edmonton, Alberta T6A 2V8
CANADA

Title of Thesis — Titre de la thèse: Validation of a growth hormone radioimmunoassay and its application to neuroendocrine studies in the goldfish, *Carassius auratus*

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VALIDATION OF A GROWTH HORMONE RADIOIMMUNOASSAY AND ITS APPLICATION TO NEUROENDOCRINE STUDIES IN THE GOLDFISH, *CARASSIUS AURATUS*

by

© ALAN FRANK COOK

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(Signed) Alan Frank Cook

PERMANENT ADDRESS:

10236 - 71 Street
Edmonton, Alberta

DATED October 7, 1981

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Validation of a growth hormone radioimmunoassay and its application to neuroendocrine studies in the goldfish, *Carassius auratus*," submitted by Alan Frank Cook in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

R. E. Peter
....
Supervisor

....

W. C. Mackay
....

S. K. Malhotra
....

L. Wang
....

Charles S. Nicoll
....
External Examiner

Date 7 October 1981

This thesis is dedicated to my parents
for their unwavering love, support and
guidance.

ABSTRACT

The present study concerned the development and validation of a radioimmunoassay (RIA) suitable for the measurement of serum immunoreactive growth hormone (ir GH) levels in the goldfish, *Carassius auratus*. The carp GH (cGH) used in the RIA was capable of promoting marked increases in growth rates of goldfish; in addition, a preliminary experiment suggested that the rabbit anti-cGH serum used in the RIA bound to endogenous circulating goldfish GH. The cGH RIA provided sensitive and reproducible measurements of goldfish serum ir GH levels. Extensive specificity testing strongly suggested that only goldfish GH reacts in this cGH RIA.

The cGH RIA was used to measure goldfish serum ir GH levels in a variety of physiological studies. Synthetic mammalian somatostatin (SRIF) was found to decrease serum ir GH levels in goldfish; two injections of SRIF given 12 hr apart resulted in significant decreases in serum ir GH levels at 1.5 and 6 hr following the second injection. In addition, there was a marked post-inhibitory rebound in serum ir GH levels at 24 hr following the second injection.

A series of experiments presented in Chapter 2 demonstrated that serum ir GH levels in female goldfish can be altered by systemic injections of a variety of catecholamines or drugs which alter the synthesis or action of catecholamines. The effect of a single intraperitoneal injection of NE on serum ir GH levels varied depending on time of year; experiments conducted between November and February suggested that NE increased GH secretion whereas in May and June NE

decreased serum ir GH levels. Additional experiments suggested that injections of L-DOPA caused dose-dependent increases in serum ir GH levels of female goldfish by decarboxylation to DA within the central nervous system.

Results presented in Chapter 3 demonstrated that lesioning the nucleus preopticus periventricularis (NPP) in goldfish resulted in both increased serum ir GH levels and increased body weight increments at 4 weeks post-lesioning. Since the NPP is a major site of SRIF immunoreactivity in another teleost species, it is possible that the NPP lesions in goldfish destroyed somatostatinergic neurons, thereby removing the SRIF inhibitory influence on the GH secretory cells.

Although a series of experiments presented in Chapter 4 provided no evidence for a circadian rhythm in GH secretion, serum ir GH levels were found to fluctuate abruptly (marked increases or decreases occurring over a 20 to 30 min interval) suggesting pulsatile GH release in the goldfish. Serum ir GH levels were found to increase progressively when groups of fish were sampled in February, April and August. Since both time of year and the experimental light-dark cycle were changed concurrently in these experiments it was not possible to determine whether these, or other factors, were responsible for the observed differences. Nevertheless, it is relevant to note that serum ir GH levels were greatest in the summer months, at the time of year when many spring-spawning teleost fishes grow most rapidly.

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GENERAL INTRODUCTION

The original studies of teleost growth hormone (GH) demonstrated that hypophysectomy results in a cessation of growth in *Fundulus heteroclitus*,and that resumption of growth could be induced by replacement therapy with GH purified from *Pollachius viriens* or *Urophycis tenius* pituitaries (Pickford and Thompson, 1948; Pickford, 1953a,b, 1954, 1959; Pickford *et al.*, 1959; Wilhelmi, 1955). Additional studies using the hyphophysectomized (hypox) male *F. heteroclitus* demonstrated the growth-promoting activity of fish and bovine GH (bGH), but not of human or simian GH, in this teleost fish (Pickford *et al.*, 1959), suggesting some species specificity in the action of GH. The original demonstration of a teleost GH (Pickford, 1953a,b, 1954) has been confirmed and supported many times with physiological (for review: Donaldson *et al.*, 1979) and histophysiological studies (for review: Ball and Baker, 1969; Sage and Bern, 1971: Schreibman *et al.*, 1973).

More recently, Farmer *et al.* (1976) and Clarke *et al.* (1977) have described the purification and properties of a GH prepared from tilapia (*Sarotherodon mossambicus*) pituitaries. Highly purified tilapia GH (tGH) behaved identically to various tetrapod GHs on Sephadex G-100, in the ultracentrifuge, and in disc gel electrophoresis, but was active only at very high doses in the rat tibia test (Farmer *et al.*, 1976). Clarke *et al.* (1977) demonstrated that tGH promoted increases in both weight and length in intact tilapia and sockeye salmon. Although unable to demonstrate a GH dose-response relationship, Clarke *et al.* (1977) concluded that bGH and tGH were approximately equipotent in the tilapia and sockeye salmon, and that the minimum effective dose of bGH and tGH to enhance somatic growth in sockeye salmon was about 0.30 to 0.40 μg GH/g body weight (BWt). Farmer *et al.* (1976) also developed a radioimmunoassay (RIA) for tGH and demonstrated that tilapia prolactin (tPRL) did not exhibit significant cross-reactivity. A significant cross-reaction was observed with pituitary extract from the perch, although the displacement curve was not parallel with the tGH standard, indicating GH immunochemical differences between these teleosts (Farmer *et al.*, 1976). The tGH RIA has not yet been used to quantify serum or pituitary GH levels in fishes.

As part of a continuing investigation of the isolation of salmon hormones, Idler *et al.* (1978), Komourdjian and Idler (1977, 1979) and Komourdjian *et al.* (1978) characterized the biochemical, immunological and biological properties of a salmon GH (oGH) prepared from chum salmon (*Oncorhynchus keta)* pituitaries. The oGH, purified by a procedure similar to that employed by Farmer *et al.* (1976), was found to stimulate linear growth in hypox rainbow trout (Komourdjian and Idler, 1979). Unfortunately, Komourdjian and Idler (1979) did not indicate the weight-specific dose of oGH used in their study. Furthermore, while these authors suggested that oGH was about two magnitudes more active than the porcine GH used as a reference in their studies, the experimental protocol used for their bioassay prevents definitive assessment of the biological activity of these GHs. It is relevant to note that two additional fractions from the oGH purification also had significant growth-promoting activity (also see below) and that salmon PRL (Idler *et al.*, 1978) was devoid of somatotropic activity (Komourdjian and Idler, 1979). Using an immunocytological technique, a rabbit antiserum generated against oGH was specifically localized to only the presumptive GH cells of salmon pituitaries (Komourdjian and Idler, 1979), suggesting antigenic differences between the salmon GH and PRL similar to that of the tilapia protein hormones (Farmer *et al.*, 1976). However, the significance of immunoreactive material in several growth-promoting side fractions from the oGH purification was not determined (Komourdjian and Idler, unpublished, cited in Komourdjian and Idler, 1979). There have been no published accounts of serum or pituitary GH measurements in fishes, using the oGH and anti-oGH sera developed by Idler *et al.* (1978) and Komourdjian and Idler (1979).

Another salmon (*Oncorhynchus tshawytscha*) GH fraction (oncGH) had approximately 10% of the activity of bGH when bioassayed for its ability to stimulate growth in intact coho salmon (Higgs *et al.*, 1978). However, since ion-exchange or gel filtration chromatography were not included in the purification procedure (see Donaldson *et al.*, 1979) it is likely that oncGH is not as pure as other teleost GH preparations. Further studies are required to evaluate the biochemical and immunological properties of this GH preparation. Farmer *et al.* (1981) recently demonstrated that a highly purified GH, isolated from pituitaries of the sturgeon (*Acipenser güldenstädti*), had significant potency in the rat tibia test. Although biochemical data suggest that the sturgeon GH (sGH) is similar to the other teleost and tetrapod GHs (Farmer *et al.*, 1981), it was not determined if sGH could stimulate growth in the sturgeon or other fishes.

The general paucity of information on the physiology of GH in teleosts is due, in part, to the lack of a simple and sensitive validated assay system. Measurements of teleost pituitary GH were attempted in the early studies of Swift and Pickford (1962a,b, 1965) in which hypox *Fundulus* were used to bioassay perch pituitary samples collected at various times of the year. It is likely that the lack of sensitivity and difficulty in evaluating the specificity of this, and other teleost GH bioassays, in addition to the length of time required to complete the assay (see Kayes, 1979), tend to preclude their routine application in the measurement of teleost pituitary and serum GH measurements. A number of studies have utilized densitometry to estimate GH concentrations following polyacrylamide gel electrophoresis (PAGE) separation of eel, guppy and trout pituitary homogenates or culture media (Baker and Ingleton, 1973, 1975; Ingleton *et al.*, 1973; Wigham *et al.*, 1975; Hall and Chadwick, 1978, 1979). The specificity of this assay system is supported only by the finding that antisera made against the two presumed GH bands after PAGE is localized to the acidophil cells of the eel proximal pars distalis (PPD) (Ingleton and Stribley, 1975); there is no evidence, however, demonstrating the growth-promoting activity of the 'GH' bands. In addition, a heterologous RIA employing radioiodinated ovine GH and antibody to ovine GH was used to estimate the relative amounts of cross-reacting antigen from serum or plasma samples of sockeye, kokanee and coho salmon (McKeown and van Overbeeke, 1972; Leatherland *et al.*, 1974; McKeown *et al.*, 1976) and goldfish

(Peter *et al.*, 1976; Peter and McKeown, unpublished results). Although these authors demonstrated parallelism between dilutions of fish plasma and pituitary extract, and showed that serum from hypox goldfish had no cross-reactivity in this heterologous RIA (McKeown, personal communication), no indications of assay precision or hormone specificity were provided. These and other limitations prevent the identification of the substance(s) measured by the heterologous RIA as a fish GH (see Nicoll, 1975). Fryer (1979) used tGH and tilapia liver membrane fractions in a radioreceptor assay (RRA) for tGH. While a variety of vertebrate GH and PRL preparations caused appreciable displacement of radiolabeled tGH from the liver membrane fraction, their displacement curves were not parallel with the purified tGH and were evident only at high concentrations (Fryer, 1979). The tGH RRA (Fryer, 1979) has been used to demonstrate that somatostatin (SRIF) can inhibit the release of GH from the pituitary glands of tilapia (Fryer *et al.*, 1979). It is likely, however, that the relatively small amount of specific binding (5 to 10%) of tGH in the RRA (Fryer, 1979) precludes the use of this assay for serum GH measurements.

The purpose of the present study was to develop a GH RIA, using a purified carp GH (cGH) and an antibody to cGH, suitable for the measurement of circulating GH levels in the carp and goldfish. Due to the nature of RIA, emphasis will be placed on validation of the GH RIA using the methods enumerated by Nicoll (1975). Thorough validation of the cGH RIA, including quantification of precision, sensitivity and specificity, are of the utmost importance. Physiological studies involving the application of the cGH RIA to the measurement of serum immunoreactive GH (ir GH) levels in the goldfish will be designed to both supplement the standard methods of RIA validation and to provide original information regarding circulating GH levels in a teleost, using a validated technique. The development and validation of the cGH RIA and the effects of SRIF and monoamines on serum ir GH levels in the goldfish are presented in Chapters 1 and 2, respectively. Chapters 3 and 4 describe investigations of the hypothalamic control of GH secretion, and studies on seasonal and daily variations of serum ir GH levels, respectively.

Chapter 1. DEVELOPMENT AND VALIDATION OF A CARP GROWTH HORMONE RADIOIMMUNOASSAY

INTRODUCTION

While a large number of studies have concerned the endocrine control of growth in fishes (for review: Donaldson *et al.*, 1979), few studies contribute directly to our understanding of the regulation of secretion of GH and the changes in blood levels of GH in teleosts. The factors responsible for this limited progress include a scarcity of fish GH preparations and the lack of an assay suitable for the measurement of serum or pituitary GH levels. Although biochemical, immunological and biological studies have been done on GH preparations from tilapia, *Sarotherodon mossambicus* (Farmer *et al.*, 1976; Clarke *et al.*, 1977; Fryer *et al.*, 1979; Fryer, 1979), chum salmon, *Oncorhyncus keta* (Idler *et al.*, 1978; Komourdjian and Idler, 1979) and sturgeon, *Acipenser güldenstädti* pituitaries (Farmer *et al.*, 1981), a fully validated RIA for application in physiological studies has not been developed using these GH preparations. Serum measurements have been made from both salmon and goldfish samples using a heterologous RIA (McKeown and van Overbeeke, 1972; Peter *et al.*, 1976; Peter and McKeown, unpublished results). However, without rigorous assay validation (for review: Nicoll, 1975), the substance(s) measured in the heterologous RIA remain unidentified. Recently, Fryer *et al.* (1979) demonstrated the suitability of a RRA for the measurement of GH released into the medium from tilapia pituitaries cultured *in vitro*. However, for technical reasons this RRA is not suitable for measurement of serum or plasma levels of GH. To date there have not been any published accounts of measurements of serum or plasma GH levels in teleosts.

The present chapter describes the biological and immunological properties of a GH prepared from carp (*Cyprinus carpio*) pituitaries by Dr. S.W. Farmer (Hormone Research Laboratory, University of California, San Francisco). Data provided by Dr. Farmer concerning the method of purification and biochemical properties of the cGH are included in APPENDIX I. In addition, the cGH was used to develop a RIA suitable for the measurement of both serum and pituitary GH levels in carp and goldfish, *Carassius auratus*. An important objective of the present study was to demonstrate that the cGH RIA provides sensitive and reproducible measurements of endogenous GH concentrations free from interference by other hormonal and non-hormonal substances.

MATERIALS AND METHODS

I. Iodination of Carp Growth Hormone

The cGH, purified as outlined in APPENDIX I, was iodinated using a modification of the method of Thorell and Johansson (1971). One mCi $Na^{125}I$ (Edmonton Radiopharmaceutical Centre, Edmonton, Alberta) in 50 µl of 0.5 M phosphate buffer (pH 7.4), 27 µg lactoperoxidase (45.7 IU/mg, Calbiochem-Behring Corp., La Jolla, CA) in 10 µl of 0.05 M phosphate buffer and 10 µl of 0.003% H_2O_2 were added to 5 µg of cGH in 5 µl of 0.05 M phosphate buffer and incubated at room temperature for 5 min with constant agitation. Two additional 10 µl aliquots of 0.003% H_2O_2 were added at 5 min intervals, and the reaction was terminated after 15 min by dilution with 500 µl of 0.05 M phosphate buffer.

Unreacted iodide and damaged proteins were separated from intact ^{125}I-cGH by gel filtration on a Sephadex G-50 (Fine) column (1.1 X 10 cm). The iodination mixture was eluted with 0.08 M barbital buffer (pH 8.6) and 1 ml fractions were collected. The specific activity of the ^{125}I-cGH varied between 110 and 175 µCi/µg. The ^{125}I-cGH was stable for about 1 week when stored at 4°C. The useful life of the ^{125}I-cGH for RIA tracer was extended beyond this period by rechromatography of undiluted ^{125}I-cGH on Sephadex G-100 (1.1 X 10 cm).

II. Radioimmunoassay Procedure

RIA was performed using a double antibody method under disequilibrium conditions. All dilutions were made with 0.08 M sodium barbital buffer, pH 8.6, containing 0.5% bovine serum albumin (BSA).

Glass tubes (12 x 75 mm) were used for incubation. Twenty-five μl of sample or standard cGH were added to 100 μl of rabbit anti-cGH serum (see APPENDIX I; 1:6000 initial dilution) containing 2.5% normal rabbit serum (NRS). After 24 hr incubation at 5°C, 100 μl of ^{125}I-cGH (9 to 11 x 10^3 cpm) was added to each tube and incubation continued for 24 hr at 5°C. Precipitation of the antibody-bound hormone was effected by addition of 200 μl of a 1:20 initial dilution of goat anti-rabbit gamma-globulin (GARGG; Antibodies Inc., Davis, CA, U.S.A.) and incubation overnight at 5°C. The tubes were then centrifuged for 30 min at 2000 rpm, and the supernatant decanted by inversion and gentle blotting on absorbent cotton. The bound fraction was then counted in an automatic gamma scintillation counter (Model MS 588, Micromedic Systems Inc. Ltd., Horsham, PA). All values obtained were corrected for nonspecific binding of the labeled hormone to the immunoprecipitate in which excess unlabeled hormone was substituted for the sample or standard. RIA results were analyzed using a weighted regression of a log-logit plot of bound ^{125}I-cGH/bound ^{125}I-cGH in the absence of unlabeled cGH (B/Bo), (Midgley *et al.*, 1969), after correction for nonspecific binding as outlined above. All samples and standards were assayed in duplicate. For statistical comparison of slopes of RIA inhibition curves from serial dilutions of serum or hormones with that of the cGH standard, the Student's t-test (2-tailed) was used. RIA inhibition curves with 4 or more duplicate values between 20 and 80% B/Bo were used for statistical testing of parallelism, when possible.

III. Molecular Heterogeneity of Immunoreactive Growth Hormone in Goldfish Serum and Pituitary Homogenate

The heterogeneity of circulating GH was studied by gel filtration on Sephadex G-100 (Fine) of several pooled serum samples which were either untreated, treated with NRS, or immunoadsorbed with rabbit anti-cGH serum. Immunoadsorbtion was performed by incubating 50 µl of goldfish serum with 100 µl of rabbit anti-cGH serum for 24 hr at 5°C. A control procedure was carried out in an identical manner in which the rabbit anti-cGH serum was substituted with NRS. Separation of antibody-bound hormone was accomplished by incubation for 24 hr at 5°C with a 1:5 dilution of GARGG, followed by centrifugation as described for the RIA. The supernatants were pooled to constitute a volume of 500 µl prior to gel filtration. Two ml of untreated serum or 0.5 ml of supernatant from the treated sera (see above) were applied to a 1.1 x 10 cm column of Sephadex G-100 and eluted with 0.08 M barbital buffer, pH 7.6. Dextran blue, ^{125}I, and ^{125}I-cGH were added to the plasma as markers; 1 ml fractions of eluate were collected and assayed for ir GH. Goldfish pituitary homogenates were chromatographed on Concanavaline A-Sepharose 4-B and the eluate collected in 1 ml fractions for RIA, to assess the amount and nature of the pituitary material(s) cross-reacting in the cGH RIA. Details of the Concanavaline A separation have been described previously (Cook and Peter, 1980).

IV. Source of Hormones and Fish Serum

The mammalian hormones used in the specificity testing of the cGH RIA and in the GH bioassay were supplied by the National Institute of Arthritis and Metabolic Disease, National Institute of Health (Bethesda, MD, U.S.A.). The carp gonadotropin (GTH) and goldfish PRL were generously provided by Drs. B. Breton (Laboratoire de Physiologie des Poissons, Institut National de la Recherche Agronomique, Campus de Beaulieu, Rennes, France) and V. de Vlaming (University of California, Davis, CA, U.S.A.), respectively. Sera from coho salmon (*Oncorhynchus kisutch*) and from common carp (*Cyprinus carpio*) were generously supplied by Drs. E.M. Donaldson (Fisheries and Marine Service, West Vancouver, B.C., Canada) and K. Berniarz (Akademia Rolnicaz, Krakow, Poland), respectively. Sera from other teleost species were obtained from fish maintained at the Department of Zoology, University of Alberta, Edmonton, Canada.

V. Bioassay of Carp Growth Hormone

Goldfish, *Carassius auratus*, of the common or comet varieties were purchased from Grassyfork Fisheries Ltd., Martinsville, Indiana. All fish were maintained for at least 2 weeks in 1500 ℓ flow-through aquaria under a simulated natural photoperiod (Edmonton, Alberta, Canada) prior to use in the bioassay. During this period the water temperature was 15 ± 1°C and the fish were fed a commercial trout chow (Ewos) twice per day. At the beginning of the experimental period, the fish were anaesthetized with 2-phenoxyethanol (Syndel Laboratories, Vancouver, B.C., Canada), and tagged on the operculum with size 1 Monel tags (National Band and Tag Co., Newport, KY, U.S.A.). The fish were each weighed to the nearest decigram (initial BWt = 12.55 ± 0.46 g, $\overline{X}$ ± SE) immediately after tagging and at 6 d intervals throughout the experimental period, commencing 10 d after the initial weighing. All weight measurements were made, after gentle blotting on absorbent towelling, between 08:00 and 09:00 hr, prior to the first feeding of the day, to reduce the contribution to variability in weight of surface and stomach contents, respectively. Preliminary experiments indicated that changes in BWt were more responsive to hormone therapy than length increments over the relatively short duration of hormone therapy (A.F. Cook, unpublished results). During the experimental period all fish were maintained in a 296 ℓ flow-through aquarium under a 16L:8D light-dark cycle (lights on at 08:00 hr) at 15 ± 1°C. Feeding was by an automatic feeder adjusted to deliver approximately 6 g Ewos (size 5P) pellets over 3.5 min, at 5 separate times during each photophase. Tanks were cleaned daily at 08:00 hr throughout the experiment.

The vehicle for hormone injections and as a control injection was teleost saline (Burnstock, 1958) supplemented with 250 U of penicillin-G (Sigma, St. Louis, MO, U.S.A.) per ml, final solution adjusted to pH 9.5 with NaOH. All injections were made intraperitoneally (ip) with a 250 μl Hamilton syringe fitted with a 27 gauge needle. Each fish received 4 injections of vehicle at 3 d intervals, beginning with the first weighing after tagging. After the pretreatment control period, the fish were randomly distributed among 5 groups to receive a total of 6 injections at 3 d intervals of either vehicle, bGH (0.5, 1.0, 5.0 μg/g BWt), or cGH (1 μg/g BWt). A post-hormone therapy control period consisted of a further 4 injections of vehicle to all fish, again at 3 d intervals; injection volumes were 5 μl/g BWt.

Instantaneous relative growth rates were calculated according to the equation $\frac{(W_T - W_t) \times 100}{(W_T)\ (T - t)}$ where W_T and W_t represent total BWt at times T and t, respectively (Ricker, 1979). After normalization of growth rates using a logarithmic transformation, results were analyzed by analysis of variance and Duncan's multiple range test (Steel and Torrie, 1960) and either the Student's t-test or, when variances were non-homogenous, the U-test (Steel and Torrie, 1960). At the end of the experiment all fish were killed with excess anaesthetic and the gonosomatic index (GSI) was calculated according to Cook and Peter (1980); the fish were then dried to a constant weight at 110°C for determination of per cent moisture.

VI. Immunohistochemistry

Goldfish were killed in excess anaesthetic, and the pituitaries were quickly removed and placed in fixative consisting of 6.25% glutaraldehyde in phosphate buffer (pH 7.4). The pars distalis (PD) was separated from the neurointermediate lobe before being cut into pieces approximately 1 mm^3 in size. The PD fragments were fixed for 20 min before washing with phosphate buffer for 30 min; post-fixation for 45 min was in 1% OsO_4 in barbital buffer (pH 7.4). After washing, tissues were dehydrated in increasing concentrations of ethanol, and embedded in Epon 812 or LX 812. Sections were cut on glass knives and mounted on uncoated copper or nickel grids. The sections on copper grids were stained with uranyl acetate and lead citrate according to Reynolds (1963), whereas the sections on nickel grids were stained with the immunohistochemical procedure (see below). In some cases, matching serial sections were cut onto copper and nickel grids in order to correlate the general ultrastructural appearance with antigen localization of the same cell. For immunohistochemistry, a modification of the procedure of Sternberger *et al.* (1970) and Moriarty (1973) was employed. Prior to staining, grids were etched in 10% aqueous H_2O_2 for 10 min and then rinsed in double distilled deionized water (DDD). Grids were then placed in a 1:30 dilution of normal goat serum (NGS) for 5 min, for control of non-specific adsorption of proteins on the tissue sections, and then incubated for 6 hr at 37°C in a 1:6000 dilution of rabbit anti-cGH serum. The grids were then placed in a solution of GARGG diluted 1:5 (see RIA procedure) for 10 min. Lyophilized peroxidase-anti-peroxidase complex (PAP) (Bionetics Laboratory Products, Kensington, MD, U.S.A.) was diluted 1:10 and stored at 6°C for not more than 5 d prior to use. After 10 min in the PAP solution and after staining in each of the above solutions, grids were rinsed in Tris-phosphate-buffered saline (0.05 M Tris, 0.001 M phosphate, 0.015 M NaCl; pH 7.6) (Tris-PBS) containing 1% NGS. The peroxidase was then allowed to react with the substrates 3, 3'-diaminobenzidine (DAB) (Sigma) and H_2O_2 for 10 min with gentle stirring. The DAB solution was made immediately prior to use by mixing 22 mg DAB with 175 ml Tris-PBS and 1.5 ml 0.3% H_2O_2. After a final DDD wash for 30 min, sections were stained with 2% OsO_4 for 30 min. Tris-PBS was used as the diluent for all of the reagents used in the immunocytochemical procedure. All solutions with the exception of H_2O_2, PAP and OsO_4 were filtered (0.22 μ pore, Millipore Corp., MA, U.S.A.) prior to use. Examination of the tissues was done with a Philips 301 electron microscope.

The specificity of the stain was tested by substitution of the following solutions for the primary antiserum (rabbit anti-cGH serum) in the staining procedure: (1) a 1:6000 dilution of antiserum adsorbed for 24 hr at 20°C with either cGH or goldfish PRL (28.6 μg/ml) or (2) NRS diluted 1:6000. As method controls, Tris-PBS was, in separate experiments, substituted for the GARGG and the PAP in the staining procedure.

VII. Administration of Antisera to Carp Growth Hormone and Effects on Growth Rates in Goldfish

Prior to beginning the experiment, 20 female goldfish (initial BWt = 7.25 ± 0.21 g, $\bar{X} \pm$ SE) were acclimated for 10 d in two 60 ℓ standing-water aquaria (10 fish per tank) maintained at 21 ± 1°C under a 16L:8D light-dark cycle (lights on at 08:00 hr). Each aquarium was equipped with 2 charcoal and glass-wool corner filters and gravel bottom filters and filled with aerated dechlorinated water containing 0.2% NaCl. The saline in each aquarium was changed twice a week prior to each set of injections (see below). The fish were fed powdered Ewos trout chow at the rate of about 4% of BWt per day. Weight-specific feeding was based on the total BWt of all fish in each aquarium determined from the most recent (not greater than 7 d) weighing. The daily ration was divided among 7 to 9 separate feedings between 08:00 and 18:00 hr and feeding was withheld for 36 hr prior to weighing. Fish were weighed to the nearest decigram, after gentle blotting on absorbent towelling, at weekly intervals throughout the experiment. The type and method of anaesthesia used prior to weighing and injections is the same as for the cGH bioassay (see above).

Instantaneous relative growth rates were calculated as:

$\frac{BWt_n - BWt_{n-1}}{BWt_n} \times 100$ where BWt_n is the BWt at week n and represents the relative per cent increase per week.

After the acclimation period, all fish were injected twice weekly (50 µl per intraperitoneal injection) with undiluted NRS for a 2 week control pretreatment period. Fish which failed to grow by more than 8% per week (N=4) during the pretreatment period were not included in the remainder of the experiment, but were left in the experimental aquaria. During the subsequent 2 week treatment period, 4 fish received twice weekly injections of undiluted rabbit anti-cGH serum (50 µl per injection) and the remaining fish continued to receive NRS injections as in the pretreatment period. Four fish from the NRS group died during the treatment period of the experiment. At the end of the experiment, the fish were killed and the GSI determined as for the cGH bioassay.

Growth rates were analyzed using either the Student's paired t-test or unpaired t-test for groups with dissimilar variances (Steel and Torrie, 1960). All confidence levels reported in Table 1.4 are 2-tailed.

RESULTS

Injections of both cGH and bGH enhanced the growth rate of female goldfish after only 2 injections of a dose of 1 µg GH per g BWt (Table 1.1). During the initial treatment period (days 20 to 26), hormone-injected fish gained weight at a significantly greater rate ($p < 0.05$) compared to their treatment vehicle injection periods, and compared to the simultaneous vehicle-injected fish. In addition, growth rates were significantly elevated ($p < 0.05$) in the cGH-injected and 0.5 µg/g bGH-injected groups during days 26 to 32 of hormone therapy in comparison with their respective pretreatment periods, and compared to the simultaneous vehicle-injected group (Table 1.1). Although goldfish injected with all 3 doses of bGH gained weight at a significantly greater rate than the control groups during days 20 to 26 of the experiment, there was no evidence for a dose-response effect during this period or during days 26 to 38 of hormone therapy. The growth rates of all the groups of fish injected with GH decreased with increasing time of hormone therapy; there were no significant differences in the growth rates of any of the GH-treated fish and vehicle control fish during days 32 to 38. Towards the end of the experiment and especially during the post-treatment period (about day 36) the weight changes of many of the experimental fish were highly variable, which is reflected by the negative growth rates and large errors shown in Table 1.1. Six fish died during the experiment; 2 in the control group and 3 and 1 in the 1.0 and 5.0 µg/g bGH groups, respectively. At the end of the experiment there were no

TABLE 1.1

Effect of carp and bovine growth hormone on relative instantaneous growth rates and per cent moisture in female goldfish.

			Relative instantaneous growth rate (% increase per day)						
			pretreatment	treatment			post-treatment		moisture
		days	10-20	20-26	26-32	32-38	38-44	44-50	(%)
treatment	dose (μg/g BWt)	N							
vehicle	--	18	0.314[1] ± 0.018	0.428 ± 0.21	0.379 ± 0.13	0.551 ± 0.17	-0.344[2] ± 0.18	0.206 ± 0.12	73.38 ± 0.73
carp GH	1.0	5	0.427 ± 0.07	1.395[3,4] ± 0.22	0.902[7] ± 0.32	0.223 ± 0.40	0.030 ± 0.44	-0.310 ± 0.22	73.63 ± 0.57
bovine GH	0.5	11	0.607 ± 0.10	1.228[3] ± 0.23	0.996[7] ± 0.36	0.557 ± 0.28	-0.484[5] ± 0.54	0.291 ± 0.22	73.80 ± 0.45
bovine GH	1.0	7	0.430 ± 0.13	1.776[3] ± 0.33	0.448 ± 0.22	-0.660 ± 0.44	0.127 ± 0.18	-0.465 ± 0.10	72.79 ± 0.92
bovine GH	5.0	9	0.489 ± 0.15	0.998[6] ± 0.27	0.468 ± 0.30	0.888 ± 0.27	-0.063 ± 0.39	-0.081 ± 0.32	74.15 ± 1.21

[1] All data are $\overline{X}$ ± SE.

[2] Significantly different from vehicle control group at all other times ($p < 0.05$).

[3] Significantly different from vehicle control group ($p < 0.01$) and from pretreatment and post-treatment periods ($p < 0.01$).

[4] Significantly different from carp GH group at days 32-38 ($p < 0.05$).

[5] Significantly different from 0.5 μg bGH/g BWt group ($p < 0.01$) at all other times.

[6] Significantly different from vehicle control group ($p < 0.05$) and from pretreatment and post-treatment periods ($p < 0.01$).

[7] Significantly different from vehicle control group and from pretreatment period ($p < 0.05$).

significant differences between any of the groups in the per cent moisture content of the carcasses (Table 1.1).

Fig. 1.1 presents a typical RIA dose-response curve for cGH and serial dilutions of serum from both intact goldfish and carp, hypox goldfish, and pituitary homogenates from goldfish and carp. Displacement of antiserum-bound radioiodinated cGH by serial dilutions of intact fish serum and the carp pituitary homogenate were parallel to the cGH standard, while the goldfish pituitary homogenate was not (see MATERIALS AND METHODS for statistical testing of RIA inhibition curves). Serial dilutions of individual serum samples from 10 to 12 hypox goldfish caused a displacement which was not significantly different ($p > 0.01$) from the zero dose response, and was therefore considered not to react in the RIA.

The mean slope, intercept, and correlation coefficient ($\overline{X} \pm SE$), calculated from a weighted regression of the log-logit specific, relative binding (Midgley *et al.*, 1969) for 10 separate cGH RIAs conducted over a 3 year period, were -2.41 ± 0.12, 4.17 ± 0.22 and 0.97 ± 0.01, respectively. The midrange of the RIA calculated as the amount of cGH standard that will bind 50% of the zero-hormone standard (Skelley *et al.*, 1973) was 54.04 ± 3.69 ng cGH per ml. Under the conditions used in the present study, the RIA sensitivity, defined as the smallest amount of antigen distinguished with a 99% probability from the zero dose level (Reuter *et al.*, 1978), was 125 pg per assay tube or 5 ng per ml serum. Within-assay (Table 1.2) and between-assay (Table 1.3) reproducibility were within acceptable limits; the coefficient of variation for 4 samples assayed four or

Fig. 1.1. Representative radioimmunoassay (RIA) dose-response inhibition curves for carp growth hormone (GH), and serial dilutions of serum and crude pituitary extracts from carp and goldfish. RIA data for serial dilutions of serum obtained from hypophysectomized (hypox) goldfish are mean values of 10 to 12 fish and the vertical bars represent the standard error; all other points represent the means of duplicate determinations. The shaded area is the portion of the RIA curve that is not significantly different from the zero dose level (see MATERIALS AND METHODS).

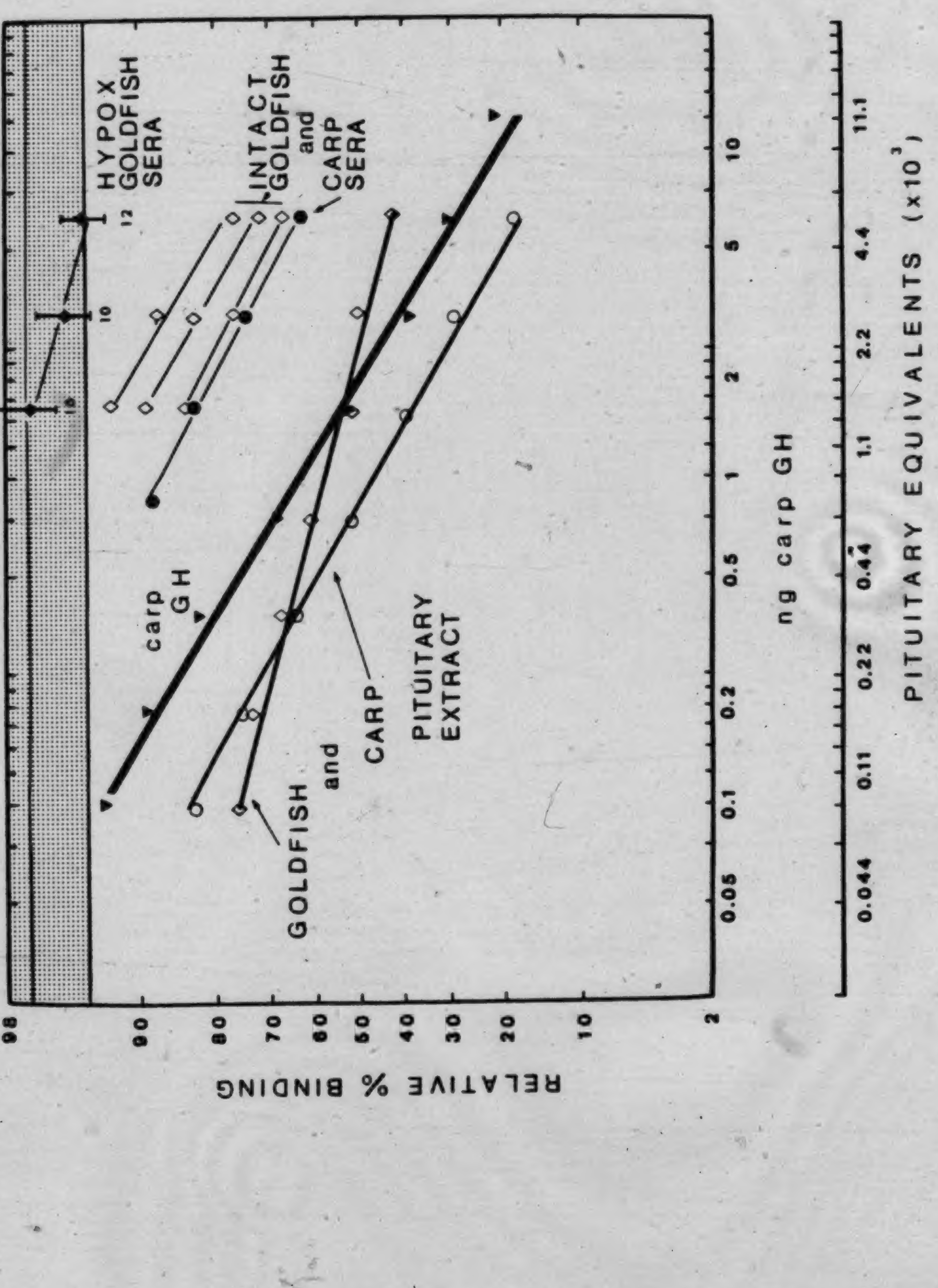

µl SERUM
5
10
20
30
15
10
12
HYPOX GOLDFISH SERA
INTACT GOLDFISH and CARP SERA
carp GH
GOLDFISH and CARP PITUITARY EXTRACT
RELATIVE % BINDING
98
90
80
70
60
50
40
30
20
10
2
ng carp GH
0.05
0.1
0.2
0.5
1
2
5
10
PITUITARY EQUIVALENTS (x10³)
0.044
0.11
0.22
0.44
1.1
2.2
4.4
11.1

TABLE 1.2

Tests of 'within-assay' reproducibility with the carp growth hormone radioimmunoassay on serum immunoreactive growth hormone (ir GH) measurements in goldfish.

Sample	A	B	C	D
(ng ir GH per ml serum)				
	26.12	43.60	12.66	34.11
	26.17	42.24	12.48	37.23
	28.79	45.27	12.22	34.79
	25.86	42.76	10.29	33.80
	---	39.25	13.03	32.02
	---	45.57	10.41	34.44
	---	44.31	---	---
Number	4	7	6	6
Mean (ng/ml)	26.74	43.28	11.85	34.40
Standard deviation (ng/ml)	1.38	2.16	1.19	1.69
Coefficient of variation (%)	5.16	4.99	10.05	4.91

TABLE 1.3

Tests of 'between-assay' reproducibility with the carp growth hormone radioimmunoassay on serum immunoreactive growth hormone (ir GH) measurements in goldfish.

Sample (ng ir-cGH per ml serum)		AA	BB	CC	DD
Assay	1	17.30	23.75	33.81	12.68
	2	19.02	15.07	28.54	16.00
	3	22.72	22.77	33.69	14.16
	4	22.42	22.03	27.87	15.39
Number		4	4	4	4
Mean (ng/ml)		20.36	20.90	30.98	14.56
Standard deviation (ng/ml)		2.64	3.95	3.21	1.47
Coefficient of variation (%)		12.97	18.90	10.36	9.95

more times in a single assay or in four or more separate assays were 6.28 ± 1.26% and 13.04 ± 2.06%, respectively.

The specificity of the cGH RIA was determined using a number of independent procedures. Fig. 1.2 illustrates that carp GTH and goldfish PRL do not significantly interfere in the cGH RIA. Although the slope of the inhibition curve for serial dilutions of goldfish PRL is not significantly different from that of the cGH standard ($p > 0.05$), goldfish PRL has only limited immunological potency in the cGH RIA, consisting of about 1% cross-reactivity. The slope of the inhibition curve by serial dilutions of carp GTH is significantly different compared to that of the cGH standard over the range of doses tested and constitutes only 5 to 8% displacement beyond the limit of sensitivity of this RIA (see Fig. 1.2 where limit of sensitivity is B/Bo = 95%). Furthermore, a second carp GTH preparation (Dr. B. Breton, Rennes, France) caused even less displacement of labeled cGH in the RIA, also in a markedly non-parallel manner (data not shown). None of the mammalian GH or PRL preparations cross-reacted in the cGH RIA (Fig. 1.2).

Fig. 1.3 illustrates the displacement curves for serum samples from a variety of teleost species. Serial dilutions of serum samples from the three Cypriniforme teleost species (carp, goldfish and sucker) and rainbow trout all gave inhibition slopes which were not significantly different from the standard cGH used in the RIA. Serum samples from the salmonid species (coho salmon and rainbow trout) caused only limited displacement of antibody-bound labeled cGH (B/Bo = 85 to 90%) in the cGH RIA. Serum from the tilapia

Fig. 1.2. Dose-response inhibition curves for carp growth hormone (GH), carp gonadotropin (GTH) and goldfish prolactin (PRL). Representative data for a carp pituitary alkaline extract (cc6e) and another intermediate fraction (cc6c) obtained during the carp GH purification are also illustrated. The radioimmunoassay data for GH and PRL from bovine (b), ovine (o) and rat (r) sources are as indicated. All data are the means of duplicate determinations.

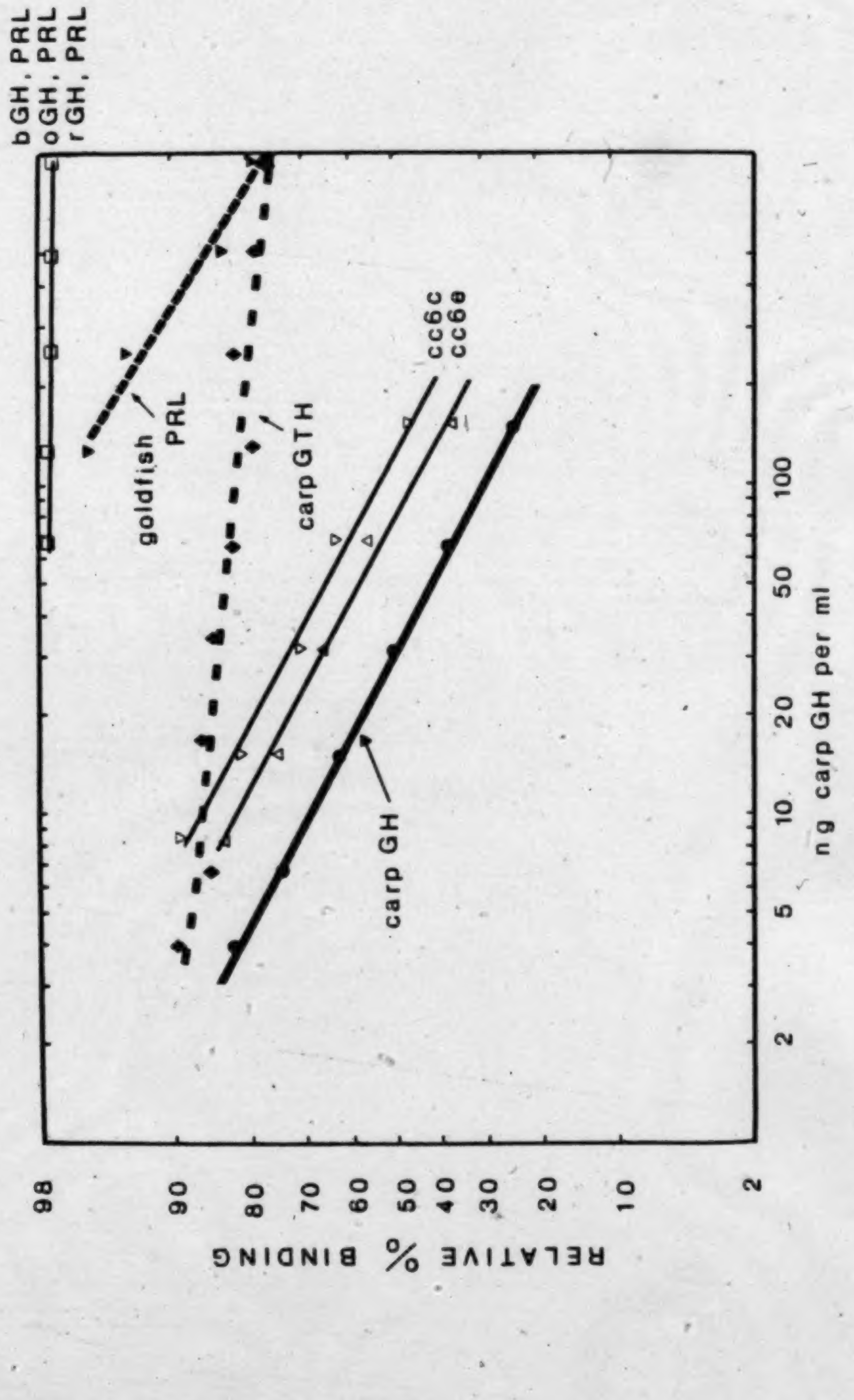
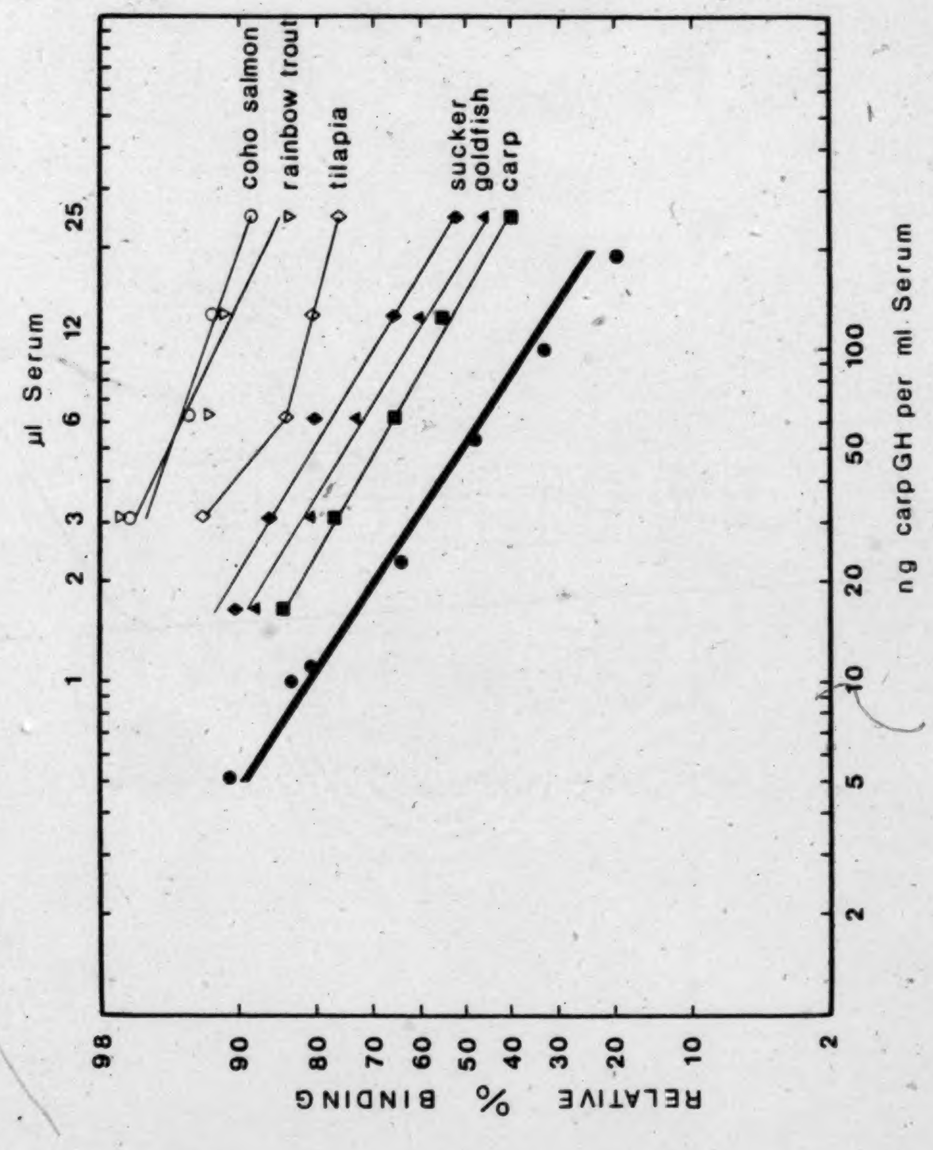

bGH, PRL
oGH, PRL
rGH, PRL
goldfish PRL
carp GTH
cc6c
cc6e
carp GH
RELATIVE % BINDING
98
90
80
70
60
50
40
30
20
10
2
ng carp GH per ml
2
5
10
20
50
100

Fig. 1.3. Dose response inhibition curves for carp growth hormone (GH) (heavy solid line) and serial dilutions of sera obtained from intact coho salmon (*Oncorhyncus kisutch*), rainbow trout (*Salmo gairdneri*), tilapia (*Sarotherodon mossambicus*), white sucker (*Catastomus commersoni*), goldfish (*Carassius auratus*) and the common carp (*Cyprinus carpio*). All data are the means of duplicate determinations.


µl Serum
1
2
3
6
12
25
coho salmon
rainbow trout
tilapia
sucker
goldfish
carp
RELATIVE % BINDING
98
90
80
70
60
50
40
30
20
10
2
2
5
10
20
50
100
ng carp GH per ml Serum

(*S. mossambicus*) showed a significant cross-reaction, although the slope of the displacement curve was not parallel with the cGH standard (Fig. 1.3).

In order to investigate the cause of the nonparallel inhibition by goldfish pituitary homogenate in the cGH RIA system (see Fig. 1.1), several goldfish pituitary homogenates were chromatographed on Concanavaline A-Sepharose (see MATERIALS AND METHODS). The results of a typical fractionation of goldfish pituitary homogenate and unlabeled cGH are shown in Fig. 1.4A. The goldfish pituitary homogenate separates into two distinct 'ir GH' fractions using this Concanavaline A separation system. The largest peak is unadsorbed to Concanavaline A, chromatographs identical to unlabeled cGH and gives parallel slopes of inhibition in the cGH RIA (Fig. 1.4B). Serial dilutions of the second Concanavaline-A adsorbed peak, consisting of 34.68 ± 5.78% ($\bar{X}$ ± SE of 4 pituitary homogenates) of the total 'ir GH' content of the pituitary homogenate, caused inhibition slopes in the cGH RIA which were significantly different compared to that of the purified cGH standard (Fig. 1.4B).

The PAP ultrastructural immunocytochemical method applied to goldfish pituitary sections revealed intense, selective staining on granules of only the somatotrope cells of the PPD (Fig. 1.5 and 1.6-1,3). Comparison of conventionally-stained sections with adjacent immunocytochemically-stained sections from the same pituitary gland (see MATERIALS AND METHODS) revealed that the stained cells were usually ovoid in shape with rounded nuclei, and with rough endoplasmic reticulum (RER) often grouped in whirls located at one cell pole or

Fig. 1.4. Fractionation of carp growth hormone (cGH) and goldfish pituitary homogenate on Concanavaline A-Sepharose 4-B (Con A). The arrow in panel A indicates the addition of 0.15 M α-methyl-D-glucopyranoside to the elution buffer. One ml fractions were collected and assayed in the cGH radioimmunoassay (RIA). RIA results of duplicate determinations of serial dilutions of selected fractions collected from the Con A separation are shown in panel B.

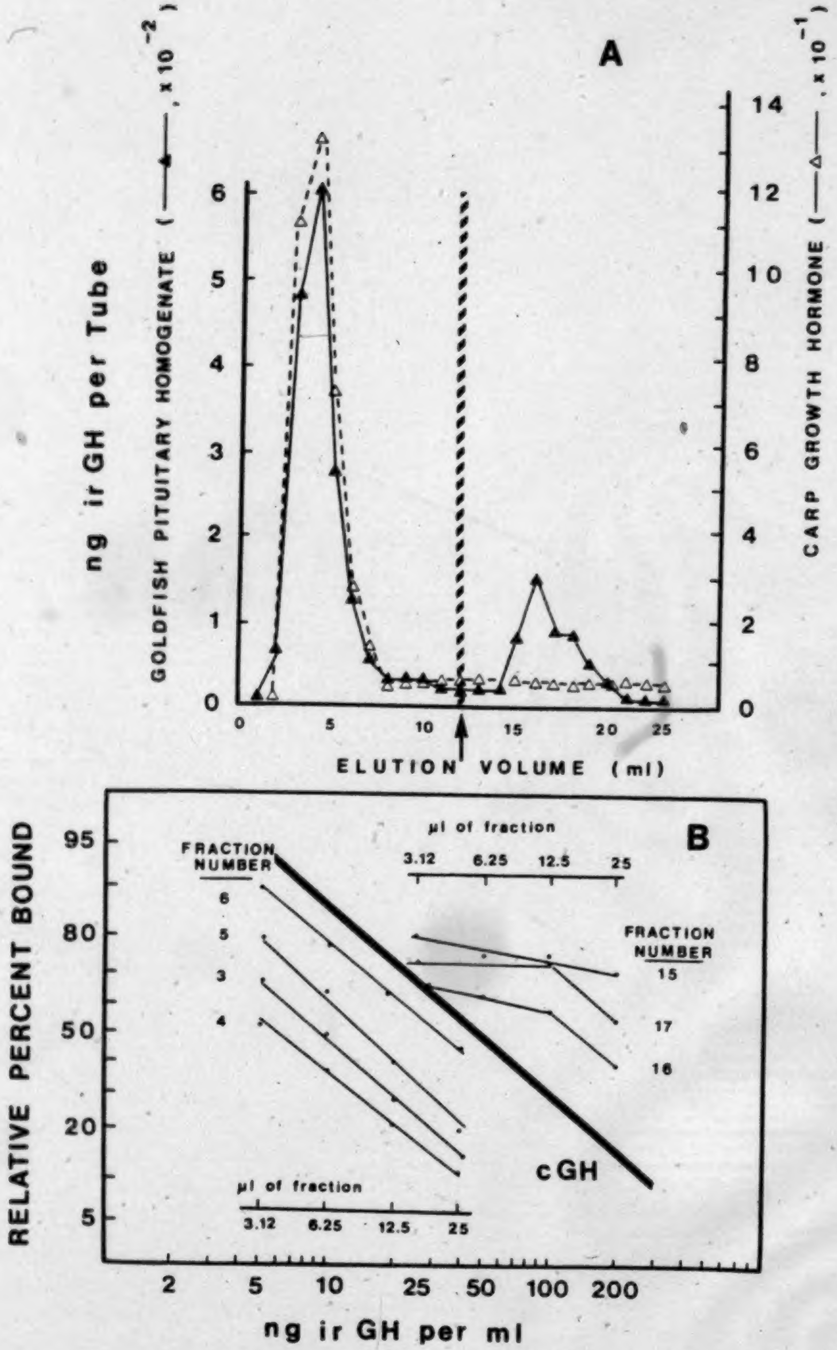

A
ng ir GH per Tube
GOLDFISH PITUITARY HOMOGENATE (—▲—, x 10^{-2})
CARP GROWTH HORMONE (—△—, x 10^{-1})
0
1
2
3
4
5
6
0
2
4
6
8
10
12
14
0
5
10
15
20
25
ELUTION VOLUME (ml)
B
RELATIVE PERCENT BOUND
95
80
50
20
5
FRACTION NUMBER
6
5
3
4
μl of fraction
3.12
6.25
12.5
25
FRACTION NUMBER
15
17
16
μl of fraction
3.12
6.25
12.5
25
cGH
2
5
10
25
50
100
200
ng ir GH per ml

Fig. 1.5. Immunocytochemical localization of growth hormone in the goldfish pituitary gland. Six positively stained somatotrope cells (GH) stand out in contrast to several unstained gonadotrope (GTH) cells. Note the larger cytoplasmic droplets (arrows) typical of GTH cells. In addition to GH and GTH cells, there is an additional cell type in this figure containing numerous smaller granules of about 1100 to 1500 Å diameter (X 3500).

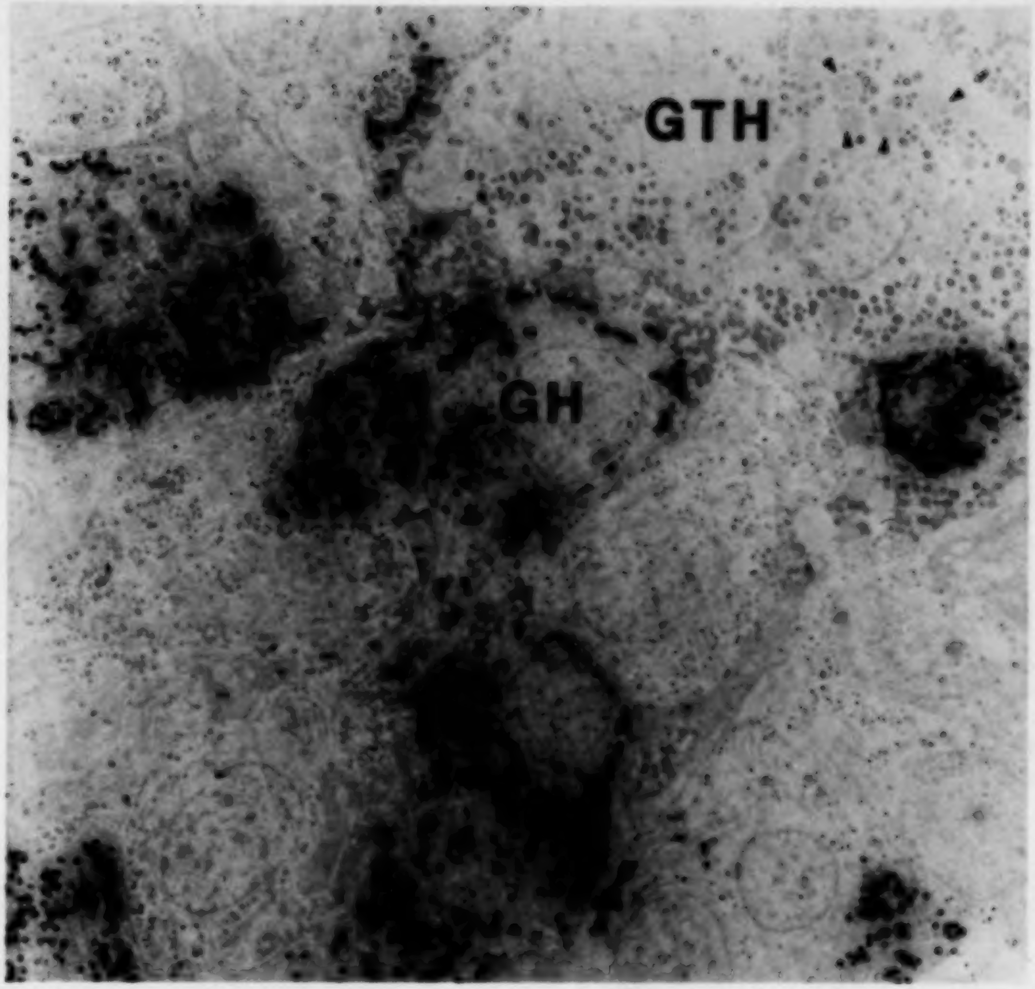
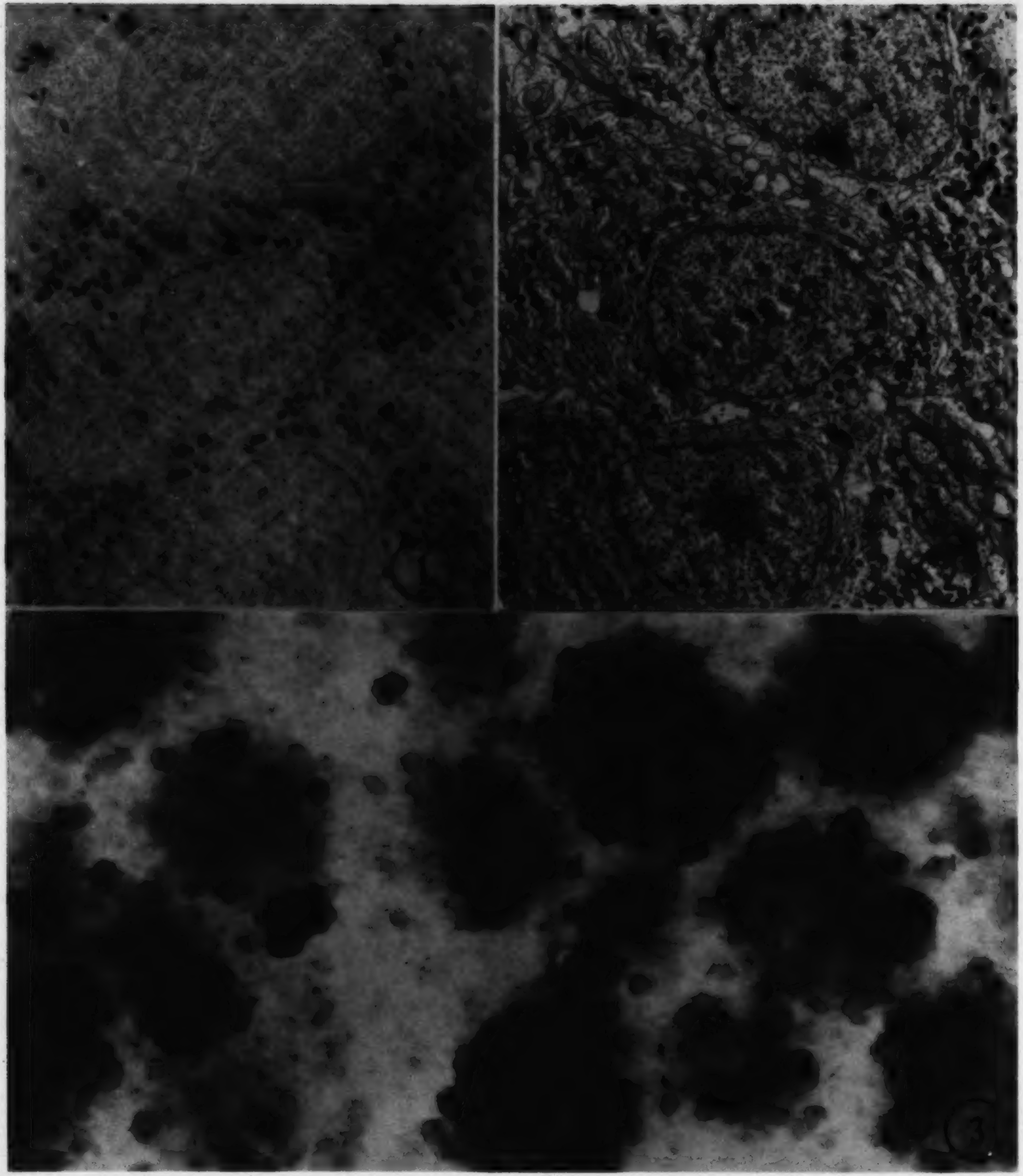

GTH
GH

Fig. 1.6-1,2. Serial sections (X 6000).

1) Immunocytochemical localization of growth hormone granules in three somatotropes of the goldfish pituitary gland.

2) The adjacent section treated with uranyl acetate and lead citrate shows the same growth hormone cells with many secretory granules and rough endoplasmic reticulum grouped concentrically around the nuclei.

Fig. 1.6-3. 3) Higher magnification shows individual peroxidase-anti-peroxidase complex molecules on growth hormone granules (X 115,000).

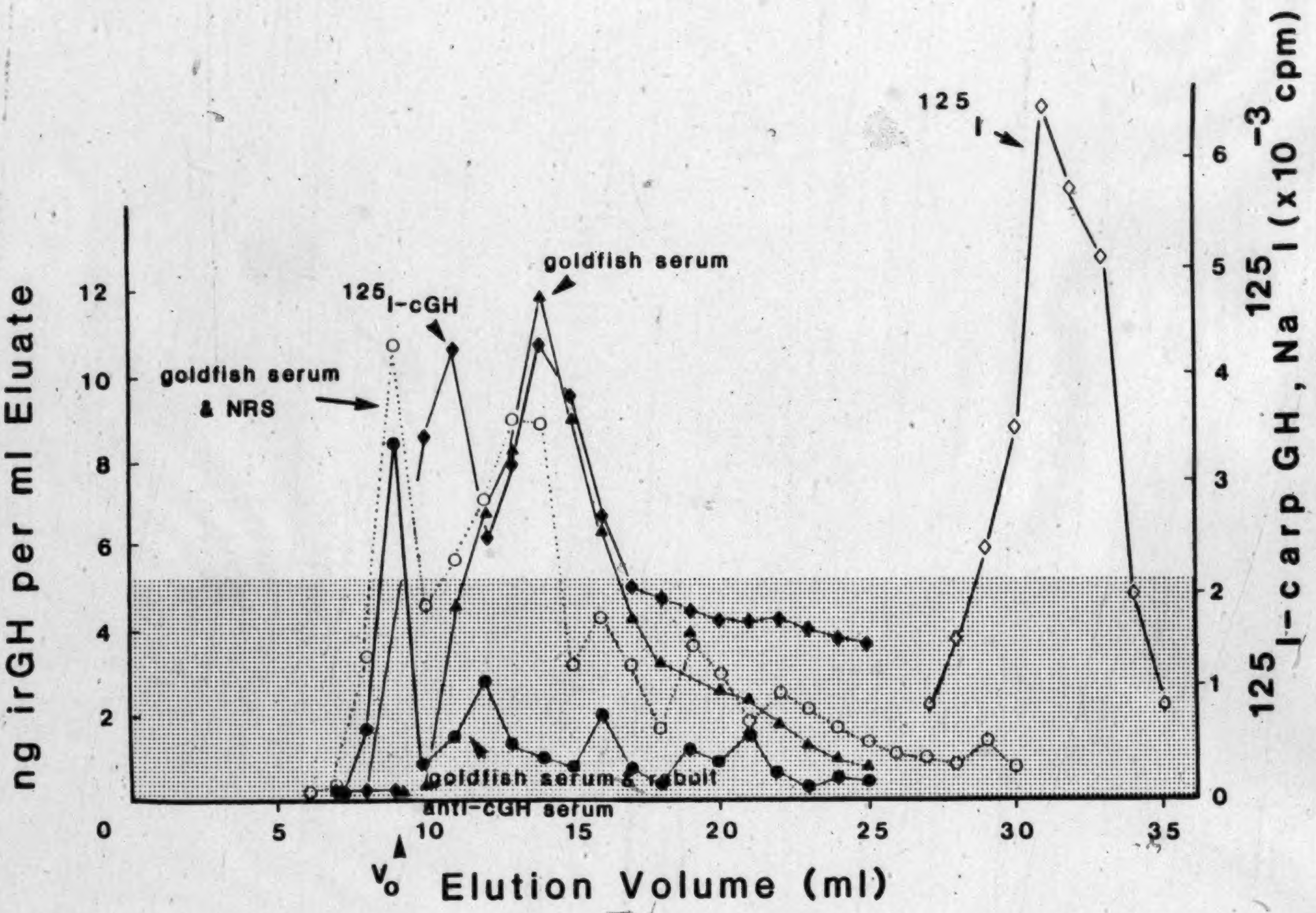

③

in concentric rings around the nucleus (Fig. 1.6-1,2). The membrane-bound cytoplasmic granules were of about 2000 to 3500 Å in diameter, comparable with that described in previous studies (Leatherland, 1972; Kaul and Vollrath, 1974) for the somatotropes of the goldfish pituitary gland. Immunostaining of GH cells with rabbit anti-cGH serum was abolished by addition of 28.6 μg/ml cGH to the primary antiserum (data not shown). In contrast, addition of an equivalent amount of goldfish PRL failed to decrease staining intensity of the GH cells. No staining was observed if NRS was substituted for the rabbit anti-cGH serum or if Tris-PBS was substituted for the PAP or GARGG.

The results of measurements of ir GH concentrations in the eluate obtained by gel filtration (Sephadex G-100) of a pooled goldfish serum sample are shown in Fig. 1.7. A single ir GH peak is evident which chromatographs with the slower eluting (i.e. smaller molecular size) of the two ^{125}I-cGH peaks. Treatment of a goldfish pooled serum sample with NRS and subsequent gel filtration resulted in 2 distinct peaks, one eluting at void volume and the other with an elution volume to void volume ratio (V_E/V_O) of 1.4 comparable with the iodinated cGH and single serum ir GH peak (V_E/V_O = 1.5) (Fig. 1.7). Chromatography of goldfish serum after rabbit anti-cGH adsorption resulted in a single 'ir GH' peak at V_0 (Fig. 1.7). In a separate experiment where immunobead GARGG (Bio-Rad Laboratories, Mississauga, Ont., Canada) was used in place of soluble GARGG, similar results were obtained, except that no peak was detected at V_0 after immunoadsorption of goldfish serum with the rabbit anti-cGH sera or treatment with NRS (data not shown).

The results of a preliminary experiment carried out in June and July, 1981, investigating the effect of rabbit anti-cGH serum on

Fig. 1.7. Gel filtration (Sephadex G-100) elution profiles are shown for goldfish serum pools which had been immunoadsorbed with rabbit anti-carp growth hormone serum (RACGH), treated with normal rabbit serum (NRS) or were untreated. For comparison, Na^{125}I and radioiodinated carp growth hormone (^{125}I-cGH, iodinated immediately prior to gel filtration, specific activity 150 μCi/μg) are also shown. The void volume, (9 ml) determined using Dextran Blue, is indicated by the arrow. For additional details see MATERIALS AND METHODS. The stippled area represents ir GH values which are less than the sensitivity of the cGH RIA (i.e. 5 ng ir GH/ml).


ng irGH per ml Eluate
0
2
4
6
8
10
12
goldfish serum & NRS
^{125}I-cGH
goldfish serum
goldfish serum & rabbit anti-cGH serum
^{125}I
0
5
10
15
20
25
30
35
V_o
Elution Volume (ml)
0
1
2
3
4
5
6
^{125}I-carp GH, Na^{125}I (x10^{-3} cpm)

relative instantaneous growth rates in sexually regressed female goldfish (GSI = 1.43 ± 0.28%) are shown in Table 1.4. During the 2 week control pretreatment period, when all fish received twice weekly ip injections of NRS, growth rates averaged over 12% per week (all fish combined) and did not differ significantly either between groups or within a group during this period (Table 1.4). During week 3, growth rates declined significantly compared to that of the previous week (week 2), in both NRS and rabbit anti-cGH serum treated goldfish. However, the fish treated with rabbit anti-cGH serum showed growth rates during week 3 which were also significantly reduced in comparison with those of week 1 (pretreatment period) and the simultaneous NRS-treated fish at week 3. During week 4 of the experiment, the mean growth rate of the rabbit anti-cGH serum treated group recovered to a value (about 9% per week) not significantly different from the pretreatment period. In a similar preliminary experiment carried out under comparable conditions, growth rates of NRS-treated fish (N=11) and fish which were uninjected (N=9) did not differ significantly over a 2 week period (data not shown).

TABLE 1.4

Effect of administration of rabbit anti-carp growth hormone serum (RA-cGH) and normal rabbit serum (NRS) on relative instantaneous growth rates in female goldfish.

		relative instantaneous growth rate (% increase per week)			
		pretreatment		post-treatment	
	weeks:	1	2	3	4
group	N				
NRS	8	11.23[1] ± 1.59	15.01[2] ± 1.24	8.01 ± 1.61	8.61 ± 1.21
RA-cGH	4	11.17 ± 0.86	12.04 ± 1.26	2.55[3] ± 1.94	9.20 ± 2.09

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[1] All data are $\overline{X}$ ± SE and all significant differences are reported below.

[2] Significantly different compared to growth rates on week 3 ($p < 0.01$) and on week 4 ($p < 0.025$) of NRS treated fish.

[3] Significantly different compared to growth rates on weeks 1 and 2 ($p < 0.05$) of RA-cGH treated fish and of NRS treated fish on week 3 ($p < 0.05$).

DISCUSSION

The cGH purified by Dr. S.W. Farmer (see APPENDIX I) and used throughout the present study shares many of the physicochemical properties of the GHs from a variety of mammalian and non-mammalian species, including similar molecular weight, similar amino acid composition, and similar behaviour in various chromatographic systems and on disc gel electrophoresis. In addition to its growth-promoting biological activity in the goldfish (see below), the cGH cross-reacted in two well-studied GH RIAs (see APPENDIX I). These assays have been shown to measure GH from a wide variety of species, including three piscine GHs: tilapia (Farmer *et al.*, 1976), shark (Hayashida, 1973) and sturgeon (Farmer *et al.*, 1981). However, it is possible that carp PRL could also cross-react since these RIAs also measure tPRL (Farmer *et al.*, 1977a). With the recent purification of a carp PRL (Idler *et al.*, 1978) and goldfish PRL (Vodicnik *et al.*,1978; V. de Vlaming, unpublished results) it may, in the future, be possible to test the teleost PRLs in these heterologous assays.

The yield of cGH was extremely low (75 mg GH/kilo, see APPENDIX I) relative to that obtained from other species (e.g. 1400 mg GH/kilo for tilapia). This may, in part, be due to the use of acetone-dried pituitaries in the present study, whereas fresh frozen pituitaries were used for the tGH purification (Farmer *et al.*, 1976). The low yield of cGH may also be related to variations in the pituitary GH content of the donor carp. Unfortunately, information on the age, sex and condition of these fish is not known. Data presented in Chapter 4 demonstrate marked seasonal variations in serum ir GH levels which, if associated with similar variations in pituitary GH levels as described for the perch (Swift and Pickford, 1965), may also explain the relatively low yield of GH obtained in the present study. It is also possible that carp 'GHs' were contained in other fractions during the purification, although the lack of material precluded their use in the bioassay employed in the present study. In support of this possibility, Komourdjian and Idler (1979) found several fractions, in addition to their salmon GH, which had significant growth-promoting activity in hypox rainbow trout (also see GENERAL INTRODUCTION).

The biochemical and biological analyses used in the present study (see APPENDIX I), including PAGE, terminal amino acid analyses and gel exclusion chromatography, all indicate that the cGH was obtained in a suitably purified form (see APPENDIX I) prior to bioassay. The biological potency of the cGH was determined by measuring its growth-promoting activity in a closely related Cypriniforme, the goldfish, *Carassius auratus*. A dose of 1 µg/g BWt of cGH caused a significant (325%) increase in instantaneous growth rate, compared to control fish, after only 2 ip injections at 3 d intervals. With both cGH and bGH, the increase in growth rates were not due to changes in water content since the per cent moisture was not different between hormone-injected and control fish. Furthermore, the increase in BWt resulting from hormone therapy was maintained after GH was withdrawn even though growth rates had returned to control levels. Unfortunately, the amount of cGH available did not permit the testing of multiple dosages to facilitate potency comparisons with bGH. Furthermore, there was no clear dose-response relationship established for bGH using the intact goldfish. Similar difficulties in determining the biological potency of teleost GH to a mammalian GH standard were also evident when both the tilapia and salmon GHs were assessed for growth-promoting activity in homologous or 'near-homologous' teleost bioassays (Clarke *et al.*, 1977; Komourdjian and Idler, 1979). Nonetheless, the results of the present study suggest that the cGH is approximately equipotent in stimulating weight increases in intact goldfish when compared to bGH. This suggests that the tGH (Farmer *et al.*, 1976) and cGH of the present study are of similar biological potency. However, it is difficult to make a direct comparison in potency with the salmon GH (Idler *et al.*, 1978) since these authors utilized hypox instead of intact rainbow trout and porcine GH as a standard in their bioassay, and did not report the weight of fish used, making it impossible to calculate weight-specific dosages (Komourdjian and Idler, 1979). Although the growth rates of the control and bGH-injected fish during the first 6 d of hormone therapy of the present study is very comparable to similar studies using intact carp (Adelman, 1977) and salmon (Higgs *et al.*, 1977, 1978), there was a reduction in response to both bGH and cGH with continued administration. This plateauing effect is similar to findings previously reported for both mammalian (Li *et al.*, 1959) and non-mammalian GHs (Wilhelmi, 1955; Kayes, 1977a; Farmer *et al.*, 1977b), tested in heterologous bioassays. It is likely that the continuous handling associated with the frequent injections of GH and morphometric measurements is stressful and is, in part, responsible for the plateauing effect. Whether or not the cGH was antigenic in the goldfish remains to be investigated, although the finding that bGH is not antigenic in several teleost species (Higgs *et al.*, 1976, 1977, 1978; Markert *et al.*, 1977) makes this explanation unlikely. In spite of the limitations of the present bioassay, there can be no doubt that the purified cGH used in this study is potent in stimulating weight gain in goldfish.

The cGH RIA developed in the present study was found to be suitable for the measurement of serum ir GH levels in the goldfish. The purified cGH showed competitive inhibition curves in the RIA which were parallel to serial dilutions of sera from a large number of goldfish. Furthermore, sera obtained from hypox goldfish did not cross-react in the RIA, indicating the pituitary or pituitary-dependent origin of the cross-reacting material in serum from goldfish with an intact pituitary gland. The cGH RIA is sensitive enough to measure ir GH in as little as 3 to 6 µl of goldfish serum, depending on the experimental conditions and season (see Chapter 4). The precision of the cGH RIA compares favourably with GH RIAs developed for other species (Schalch and Reichlin, 1966; Tsushima *et al.*, 1971; Borer and Kelch, 1978), with an overall 10% coefficient of variation (CV) (average of within and between assay CV) in the useful range of this RIA.

To date only GTH, PRL and GH have been isolated from teleost pituitaries, restricting the specificity testing of the cGH RIA to only these teleost hormones. The cross-reactivity of carp GTH in the cGH RIA was of a very minor and nonspecific nature, and is not likely due to GH contamination of the GTH preparation since the inhibition curve was virtually flat compared to the cGH standard. The finding that the goldfish PRL (see Vodicnik *et al.*, 1978) does not cross-react to an appreciable extent in this RIA is of considerable significance in view of the biological, chemical and immunological data indicating similarities between GHs and PRLs prepared from a number of species (Nicoll, 1974; Hayashida *et al.*, 1975; Farmer and Papkoff, 1979). However, results obtained using this goldfish PRL preparation must be viewed somewhat cautiously, since the biological properties of this hormone have not been fully evaluated (Vodicnik *et al.*, 1978). Nonetheless, antisera prepared against tGH (Farmer *et al.*, 1976), salmon GH (Komourdjian and Idler, 1979) and cGH (present study) do not appear to cross-react appreciably with the homologous teleost PRL, suggesting greater antigenic differences between GH and PRL in teleosts than previously suspected. Furthermore, a number of studies also have shown a clear distinction between GH and PRL biological activity in teleosts (Doneen, 1976; Clarke *et al.*, 1977; Idler *et al.*, 1978, Komourdjian and Idler, 1979). While the isolation of larger quantities of teleost GH and PRL is essential for a more precise determination of the biological activity and specificities of these hormones, it is interesting to speculate that the longer period of evolutionary history of the teleost fishes relative to that of the mammals has contributed to the greater immunochemical and biological differences between these two hormones.

Although other goldfish pituitary hormone preparations are unavailable for testing in the RIA (see above), the dilution response curves for goldfish serum samples were parallel to that obtained for the purified cGH. Moreover, ovulation, which is known to initiate a marked surge in ir GTH in the goldfish (Stacey *et al.*, 1979) had no effect on ir GH levels, and injections of dopamine, known to inhibit PRL release in the goldfish (Vodicnik *et al.*, 1978), caused either no change or a significant increase in serum ir GH levels (see Chapter 2). Taken together, the results outlined above strongly suggest that cross-reaction of PRL, GTH and other pituitary hormones in the cGH RIA is either of a very minor nature or nonexistent.

None of the mammalian GHs or PRLs that have been tested in either the cGH RIA (present study), tGH RIA (Farmer *et al.*, 1976) or tGH RRA (Fryer, 1979) have produced appreciable cross-reaction, even when assayed at very large doses. While these findings do not address the important question of assay specificity with regard to the teleost pituitary hormones (see above), they indicate major immunological differences between the mammalian and teleost GHs. In support of this, antisera produced against a variety of mammalian protein hormones show immunocytological cross-reactivity with teleost pituitary cells only at very large concentrations (e.g. 1:5 to 1:20 dilution) (Emmart *et al.*, 1966; Emmart, 1969; McKeown and van Overbeeke, 1971; Aler, 1971). The finding that teleost GH is relatively inactive in mammals (Farmer *et al.*, 1976; see GENERAL INTRODUCTION) but that mammalian GHs are approximately equipotent with teleost GH when assayed for growth induction in fishes (Clarke *et al.*, 1977; Komourdjian and Idler, 1979; present study) suggest both similarities and differences in specificity of GH activity between these vertebrate groups. In spite of the strong biochemical evidence demonstrating that the structure of GH has been conserved during evolution (for review: Farmer and Papkoff, 1979), available results do not permit generalizations regarding immunological and biological relatedness among GHs from mammalian and teleost species.

Based on the preliminary results from the RIA of serum from a variety of fish species, the present study indicates that the cGH RIA may be used for species other than goldfish. Furthermore, the finding that serum from coho salmon and tilapia, but not rainbow trout, showed slopes of inhibition in the RIA which did not parallel the cGH standard suggests that there are both immunochemical differences in circulating GH among even closely related teleost species. The finding that perch pituitary extract (Farmer *et al.*, 1976) and goldfish serum (S.W. Farmer, personal communication) do not cross-react in the tGH RIA also suggest immunochemical differences between these teleosts. Additional studies including RIA of serum from a greater variety of fish species are required to determine whether or not there is any GH immunochemical phylogenetic pattern among teleosts. Since serum from the three Cypriniforme species (carp, goldfish and sucker) and the rainbow trout showed parallel slopes of inhibition in the RIA, it is possible that valid GH measurements can be made in serum samples from these species. It is important to note however, that immunochemical parallelism between serum and the purified cGH standard provides only the initial step in determining the validity of the GH measurement by RIA. In the present study additional specificity testing was accomplished using goldfish and carp pituitary hormones in the RIA, immunohistochemistry applied to goldfish pituitary sections, biochemical separations of goldfish serum and by testing serum from hypox goldfish. Further investigations are required to determine whether or not the cGH RIA can be utilized for the measurement of serum GH levels in species other than goldfish.

In contrast to results obtained using serum, goldfish pituitary homogenates did not cause a parallel inhibition slope in the cGH RIA, which precludes the measurement of ir GH in crude goldfish pituitary extracts. However, the present study has demonstrated that the nonparallel displacement curve is due to the presence of a pituitary substance(s) that is adsorbed to Concanavaline A, which if removed using the described separation system establishes a parallel slope of inhibition. Since the other tests of specificity, including ultrastructural immunocytochemistry (see below) and RIA testing of other goldfish and carp pitutiary hormones (see above) support the validity of pituitary GH measurements in goldfish, the Concanavaline A separation may be used for the determination of goldfish pituitary ir GH levels. It is possible that the relatively small and variable contribution of the goldfish Concanavaline A-adsorbed pituitary material cross-reacting in the RIA prevented its ultrastructural localization using the PAP immunocytochemical method (see below). Alternatively, the cross-reacting material may be a component of the GH granules of somatotrophs stained by the PAP reaction which may contain a glycoprotein moiety that is not released into the circulation. It is interesting to note that in contrast to goldfish pituitary extracts, carp pituitary extracts have parallel slopes of inhibition in the cGH RIA, emphasizing the importance of carefully determining the suitability of a RIA for even closely related species.

Further testing of the specificity of the cGH RIA based on results obtained using the PAP immunocytochemical technique (Sternberger *et al.*, 1970; Moriarty, 1976) confirms and extends those obtained by RIA (see above). Identification of the presumptive somatotrope cells in the goldfish pituitary was based on the location of the cells within the pituitary and by ultrastructural criteria detailed by Leatherland (1972), Nagahama (1973) and Kaul and Vollrath (1974). Briefly, the somatotrope cells of the goldfish pituitary may be recognized by their overall oval or pyramidal shape, with a round or oval nucleus and electron dense granules which are larger than those in PRL cells, together with an extensive and polarized RER. The specificity of the staining reaction was demonstrated by the finding that absorption of the rabbit anti-carp GH serum with cGH, but not goldfish PRL, abolished the staining reaction on the GH granules. This finding is in agreement with results detailed above in which goldfish PRL was shown not to cause appreciable cross-reactivity in the cGH RIA. In addition, all methodological control procedures supported the validity of the PAP ultrastructural method using the rabbit anti-cGH serum for staining goldfish GH granules. Since only the presumed somatotrope cells of the goldfish pituitary reacted with the rabbit anti-cGH serum, the specificity of the RIA for only goldfish GH is further supported. It is also important to note that the rabbit anti-cGH serum was used at dilutions comparable with that used in the RIA (see MATERIALS AND METHODS), and that the sensitivity of this PAP technique is reported to be at least as great as for the RIA (Petrali *et al.*, 1974). It must be noted, however, that the cGH RIA is, in the present study, employed primarily for the measurement of serum, and not pituitary, ir GH levels in goldfish. If the pituitary and circulating form of GH in goldfish differ in their immunological and/or biological properities, as has been suggested for the rat (Vodian and Nicoll, 1977) and goldfish (see discussion concerning Concanavaline A separation of goldfish pituitary homogenate), then use of both the PAP technique and RIA for testing the immunological specificity of goldfish pituitary hormones may not be relevant to the question of possible cross-reactivity of circulating hormones. However, the results of the immunoadsorption of GH from goldfish and subsequent chromatography on Sephadex G-100 demonstrate the similarity, at least in terms of size, of the serum ir GH and the cGH standard. Furthermore, since serial dilutions of goldfish serum show RIA inhibition curves which parallel that of purified cGH (see above), the immunological similarity between the circulating and pituitary form of goldfish GH is supported. Without highly purified hormones isolated from goldfish serum, the results described above strongly support the specificity of the cGH RIA for measurement of serum GH in goldfish.

A preliminary experiment showed that twice weekly ip injections of anti-cGH serum caused significant decreases in growth rates in goldfish after 1 week of treatment. It is likely that neutralization of endogenous circulating goldfish GH by the rabbit anti-cGH serum is responsible for the observed reduction in growth. These results assume some significance in light of recent investigations of the relationships between circulating levels of immunoreactive and biologically active GH (Vodian and Nicoll, 1977; Russell *et al.*, 1980) which emphasize that RIA makes an immunological measurement (i.e. displacement of radiolabeled hormone from antisera) of a presumed biologically active hormone. These results, in conjunction with those of the specificity tests detailed above, indicated that endogenous goldfish GH shares immunological determinants with the cGH and the anti-cGH serum used in the cGH RIA. However, the finding that there was no significant reduction in growth rate during the second week of rabbit anti-cGH treatment is difficult to explain. It is possible that rabbit anti-cGH serum is antigenic in goldfish or that endogenous GH secretion was greatly increased after continued administration of this antiserum. Although additional studies requiring larger amounts of antiserum for injections of greater numbers of fish over a longer period of time are needed, this preliminary experiment suggests that the rabbit anti-cGH serum used in the RIA binds biologically active circulating GH in goldfish.

An attempt was also made to correlate RIA (present study) and bioassay (Adelman, 1977) estimates of GH content of pituitaries collected from two separate populations of carp at different times of the year (I. Adelman and A.F. Cook, unpublished results). However, the absence of significant seasonal variations in bioactive pituitary GH content in carp (I. Adelman, unpublished results) and the variability of the carp bioassay made it possible to establish a statistically significant correlation between the bioassay and RIA results ($r^2 = 56.4\%$, N=7, $p < 0.05$) for only one set of samples (I. Adelman and A.F. Cook, unpublished results). Unfortunately, available teleost bioassays are neither sensitive nor precise enough to measure serum GH levels. The question of whether the cGH RIA measures circulating biologically active goldfish GH is studied by the experiments described in Chapters 3 and 4. Briefly, these studies directly and indirectly establish positive relationships between the serum ir GH level and the growth rate of the goldfish. In Chapter 3, lesioning the nucleus preopticus periventricularis resulted in both an increased weight gain and serum ir GH levels in goldfish, whereas lesions not affecting weight changes had no effect on serum ir GH levels. Furthermore, serum ir GH levels show a seasonal pattern in both goldfish and suckers, with elevated circulating ir GH levels occurring after the spawning period, when growth is most rapid (see Chapter 4).

In summary, the cGH RIA described in the present chapter is both precise and sensitive and appears to measure serum ir GH free from interference from other hormonal and non-hormonal substances. Furthermore, the specificity of the cGH RIA for measurement of serum GH in goldfish has been shown in the present study, to a degree greater than that demonstrated for any other RIA for a teleost pituitary hormone (Breton *et al.*, 1972; Crim *et al.*, 1976; Farmer *et al.*, 1976; Hontela and Peter, 1978). It is apparent then that the cGH RIA provides a valid means of studying the physiology of circulating ir GH levels in goldfish.

Chapter 2. THE EFFECTS OF SOMATOSTATIN AND MONOAMINES ON SERUM GROWTH HORMONE LEVELS IN THE GOLDFISH, *CARASSIUS AURATUS*

INTRODUCTION

The participation of the catecholaminergic system of the brain in the central regulation of GH release is established in several mammalian species (Ganong, 1975; Ruch *et al.*, 1977; Edén *et al.*, 1979). Catecholamines appear to influence GH secretion by way of central sites, affecting the hypothalamic neurons which produce somatostatin (SRIF, also known as somatotropin release-inhibiting factor) and/or an as yet unidentified GH releasing factor (GHRF) (Martin, 1976; Martin *et al.*, 1978; Weiner and Ganong, 1978). For example, in the dog, administration of the amino acid precursor of catecholamines, dihydroxyphenlalanine (L-DOPA) increases GH secretion by an action inside the blood-brain barrier (Lovinger *et al.*, 1976). Considerable evidence suggests that L-DOPA increases GH secretion in the dog by its subsequent conversion to norepinephrine (NE) and not to dopamine (DA) or epinephrine (E) (for review: Weiner and Ganong, 1978). Furthermore, intraventricular injections of NE and the central acting α-agonist clonidine also increased GH levels while the α-receptor antagonist phentolamine reduced GH levels (Lovinger *et al.*, 1976). Although it is not known whether L-DOPA increases GH secretion by decreasing SRIF or increasing GHRF secretion, evidence obtained from the rat (Durand *et al.*, 1977), cat (Ruch *et al.*, 1977), baboon (Steiner *et al.*, 1978) and human (Lal *et al.*, 1975) also are consistent with an α-adrenergic mechanism for stimulating GH secretion. Since intravenous administration of anti-SRIF serum increases basal GH levels, there is at least some degree of basal SRIF secretion (Ferland *et al.*, 1977), and it may be that NE reduces SRIF release to cause the increase in GH secretion.

In contrast to the situation in mammals, very few studies have investigated the possible effects of either monoamines or SRIF on GH secretion in teleost fishes. A major obstacle in this area of comparative endocrine research has been the lack of an assay suitable for the measurement of serum GH levels in non-mammalian vertebrates. A validated RIA technique for measurement of serum GH has been reported for the bullfrog *Rana catesbeiana* (Clemons, 1976), and a radioreceptor assay (RRA) suitable for the measurement of GH released from *Sarotherodon mossambicus* pituitaries cultured *in vitro* has been described (Fryer, 1979; Fryer *et al.*, 1979). Chapter 1 describes a RIA for GH in carp, *Cyprinus carpio* and goldfish, *Carassius auratus*.

SRIF has been shown to inhibit GH released into the culture medium from pituitary glands of the teleost fish, tilapia (*S. mossambicus*), as measured by RRA (Fryer *et al.*, 1979). At concentrations of 0.1 to 2.0 μg per ml incubation medium, SRIF produced a dose-dependent inhibition of GH release; the highest concentration of SRIF (2.0 μg/ml) producing an 80% inhibition of GH release compared with control values (Fryer *et al.*, 1979). In addition, SRIF has been measured by RIA in brain extracts of *S. mossambicus* (King and Millar, 1979) and the catfish and hagfish (Vale *et al.*, 1976). Furthermore, the teleost SRIF is immunologically and chemically very similar to, if not identical with mammalian synthetic SRIF (King and Millar, 1979). Using immunocytochemical procedures Dubois *et al.* (1978, 1979) have demonstrated the presence of SRIF in the pituitary and hypothalamus of rainbow trout, *Salmo gairdneri*. While it is certain that SRIF is present in the hypothalamus of fishes and that SRIF can inhibit GH release from tilapia pituitary glands cultured *in vitro*, it is essential to determine the effect of SRIF on serum GH levels in intact fish before postulating a role for SRIF in the regulation of GH secretion in teleosts.

The present study was designed to determine the effect of several doses of SRIF on serum ir GH levels in goldfish and to determine the time-course of the response. Although several studies have employed histochemical procedures to demonstrate monoamines in the brain and hypothalamus of several species of fish (Baumgarten and Braak, 1967; Terlou *et al.*, 1978), there is no published information concerning the possible influence of monoamines on either SRIF or GH secretion in this vertebrate group. Therefore, the present study also investigates the effect of a variety of monoamines and related drugs and their interaction with SRIF on serum ir GH levels in goldfish, to gain insight into the neural regulation of teleost GH secretion.

MATERIALS AND METHODS

I. General Procedures

The source and general procedures for the handling of, and method of anaesthesia of goldfish were as described for the cGH bioassay (see Chapter 1).

II. Experiments

Somatostatin Experiments

Sexually mature male goldfish (27.38 ± 0.56 g BWt, $\overline{X}$ ± SE, Experiment 2.1; 36.77 ± 1.09 g BWt, Experiment 2.2; 32.01 ± 0.94 g BWt, Experiment 2.3) were acclimated to conditions of 12 ± 1°C and a 16L:8D light-dark cycle (lights on at 08:00 hr) for a minimum of 2 weeks. All somatostatin experiments and the combination experiment (see below) were done in May and June. The fish were fed Ewos size 5P pellets twice or three times daily at different times during the photophase, except that food was withheld on the day of, and the day prior to blood sampling (see below). At the start of each experiment the fish were individually tagged and weighed (see Chapter 1) and a pretreatment blood sample taken. The method of sampling and preparation of serum for subsequent RIA analysis were as described by Cook and Peter (1980). The initial (pretreatment) blood sample (90 to 110 µl volume) was taken from all fish at 20:30 to 21:00 hr, immediately prior to the first ip injection of either physiological saline (PS; Burnstock, 1958) or the experimental solutions described below. A second ip injection was given 12 hr later at 08:30 hr and each fish was bled at 1.5 hr in Experiment 2.1, and at 1.5, 6 and 24 hr following the second injection in

Experiments 2.2 and 2.3. After the final blood sample, the fish were killed in excess anaesthetic, weighed and the gonads were removed and weighed.

The SRIF used in Experiments 2.1 and 2.2 was synthesized by Dr. J. Rivier and generously supplied by Dr. W. Vale of the Salk Institute, La Jolla, CA, whereas for Experiment 2.3, SRIF was purchased from Beckman Instruments Inc., Palo Alto, CA. Thyrotropin-releasing hormone (TRH) and Substance-P were also obtained from Beckman Instruments Inc. All solutions were prepared immediately prior to use and the vehicle used for dissolution and injection of neuropeptides was also PS. Ip injections were administered in a volume of 10 µl/g BWt using a 250 µl Hamilton syringe fitted with a 27 gauge needle. The dosages of neuropeptides used in Experiments 2.1, 2.2 and 2.3 are included in Table 2.1 and the figure legends to Fig. 2.1 and 2.3, respectively.

Drug Experiments

Female or male goldfish were acclimated for at least two weeks to 12 ± 1^{o}C, and either a 12L:12D or 16L:8D light-dark cycle (lights on at 08:00 hr). On the day of drug administration feeding was withheld and all injections and blood sampling was performed between 08:30 and 14:30 hr. Alpha-methyl-paratyrosine ester HCl (AMPT), DA, NE (NE HCl), reserpine and L-DOPA were purchased from Sigma (St. Louis, Missouri, U.S.A.). Clonidine HCl and phentolamine HCl were kindly donated by Boehringer Ingelheim Ltd., Ridgefield, CT, U.S.A. and CIBA-GEIGY Canada Ltd., Dorval, PQ, Canada, respectively. These drugs were administered as a solution or as a suspension in a vehicle of acidified 0.7% sodium chloride with 0.1% sodium metabisulfite. Ip injections of drugs were made with a 1 ml syringe fitted with a 27 gauge needle. The times of blood sampling relative to that of injection are included in the appropriate tables (see RESULTS, Tables 2.2 to 2.10).

Combination Experiment

Sixty-seven male goldfish (24.60 ± 0.57 g BWt, $\overline{X}$ ± SE) were acclimated for 18 d in 96 ℓ flow-through aquaria (14 to 18 fish per tank) at 24 ± 2°C under a 16L:8D light-dark cycle (lights on at 08:00 hr). The fish were fed as described above and feeding was withheld on the day prior to and the day of blood sampling. To start the experiment fish were individually tagged and weighed and a pretreatment blood sample was taken as described for the SRIF experiments (see above). Immediately following, and at 12 hr after the pretreatment blood sample, each fish was injected with one of the solutions described below. SRIF (1 μg/g BWt; obtained from Boehringer Mannheim, Dorval, PQ, Canada), L-DOPA (50 μg/g BWt; Sigma), 2-(3,4 dihydroxybenzyl) 2 hydrazinopropionic acid (CARBIDOPA, 50 μg/g BWt; kindly donated by Merck Frosst Laboratories, Dorval, PQ, Canada), SRIF and L-DOPA (1 μg/g BWt and 50 μg/g BWt, respectively) and L-DOPA and CARBIDOPA (50 μg/g BWt for each drug) were all dissolved in PS immediately prior to use, and injected ip in a volume of 10 μl/g BWt as described for the SRIF experiments. Control fish were injected with an equivalent volume of PS. Post-treatment blood samples were taken at 1.5 and 24 hr following the second injection and the serum was processed for the cGH RIA in the usual manner.

III. Carp Growth Hormone Radioimmunoassay

Details of the cGH RIA are as described in Chapter 1.

IV. Statistical Analyses

Somatostatin Experiments

Analysis of variance and Duncan's multiple range test at $p < 0.01$ were used to determine differences between groups at each of the sampling times (Steel and Torrie, 1960). The raw data were normalized using a logarithmic transformation prior to statistical testing. Paired t-tests (2-tailed) were used to compare serum ir GH concentrations of pretreatment and post-treatment samples from individual fish within experimental groups (Steel and Torrie, 1960).

Drug Experiments

For comparison of the effects of a single drug on serum ir GH levels, either the Student's t-test (2-tailed) or, if the variances were significantly different as indicated by Bartlett's x^2 test, the Mann-Whitney U-test were used (Steel and Torrie, 1960). The effects of several doses of the same drug were assessed using analysis of variance and Duncan's multiple range test at $p < 0.01$.

Combination Experiment

The Student's t-test or Mann-Whitney U-test (see above) were used for comparison of mean serum ir GH levels of each treatment group with that of the vehicle control group. The ir GH data were normalized using a logarithmic transformation prior to statistical testing. The paired t-test was used to compare mean serum ir GH levels at 1.5 and 24 hr following the second injection with that of the presample value. All significance levels reported in Table 2.11 are 2-tailed.

RESULTS

Somatostatin Experiments

Table 2.1 summarizes the effects of two ip injections, given 12 hr apart, of either SRIF, TRH, or Substance-P, on serum ir GH levels in sexually mature male goldfish (GSI = 3.68 ± 0.23%) (Experiment 2.1). Of the peptides tested, only SRIF (1 μg/g BWt) had a significant effect, decreasing serum ir GH levels by 56% at 1.5 hr following the second injection (Table 2.1). Fig. 2.1 illustrates the results of Experiment 2.2, describing the time-course of inhibition of serum ir GH levels in sexually mature male goldfish (GSI = 3.39 ± 0.29%) after 2 injections of SRIF (1 μg/g BWt) given 12 hr apart. SRIF caused a significant decrease ($p < 0.01$) in serum ir GH levels compared to both presample and vehicle-injected control values at 1.5 and 6 hr following the second injection (Fig. 2.1). At 24 hr following the second injection there was a significant rebound to levels that were more than twice those of presample control values. Fig. 2.2 illustrates the effect of SRIF on serum ir GH levels in individual fish before and after SRIF treatment (Experiment 2.2, same data as Fig. 2.1). It is apparent that the inhibitory action of SRIF on mean serum ir GH levels (Fig. 2.1) is due to a decrease in the serum ir GH levels of fish that had elevated levels (ca. > 25 ng ir GH/ml serum) at the time of the pretreatment sample (Fig. 2.2). TRH (1 μg/g BWt) caused a significant increase in serum ir GH levels at 24 hr, but not at 1.5 or 6 hr after the second injection compared with both pretreatment and vehicle-control values (Fig. 2.1).

TABLE 2.1

The effect of two intraperitoneal injections of some neuropeptides given 12 hours apart on serum immunoreactive growth hormone (ir GH) levels in the goldfish at 1.5 hours following the second injection (Experiment 2.1).

treatment	dose (μg/g BWt)	N	ng ir GH per ml serum
vehicle	--	15	31.04 ± 2.41[1]
TRH[2]	1 μg/g	9	27.17 ± 4.05
SRIF[3]	1 μg/g	10	17.31 ± 2.64[4]
Substance-P	1 μg/g	10	28.38 ± 3.39

[1] All data are $\overline{X}$ ± SE.

[2] Synthetic porcine thyrotropin-releasing hormone.

[3] Synthetic linear somatostatin.

[4] Significantly different compared to vehicle control value ($p < 0.01$).

Fig. 2.1. (Experiment 2.2). The effect of synthetic linear somatostatin (SRIF, //////////////, 1.0 µg SRIF/g BWt, N=18), synthetic thyrotropin-releasing hormone (TRH, --------------, 1.0 µg TRH/g BWt, N=12), and physiological saline (PS, ——————, 5 µl/g BWt, N=18) on serum immunoreactive growth hormone (ir GH) levels in male goldfish. All fish were sampled immediately prior to the first of two intraperitoneal injections given 12 hours apart (presample, p) and at 1.5, 6 and 24 hours following the second injection. The values shown are $\overline{X}$ ± SE.

Paired t-test using log-transformed data ($p < 0.01$).

PS	p	6	24	1.5
SRIF	1.5	6	p	24
TRH	p	1.5	6	24

ANOVA and Duncan's multiple range test using log-transformed data ($p < 0.01$).

p	TRH	SRIF	PS
1.5	SRIF	PS	TRH
6	SRIF	PS	TRH
24	PS	TRH	SRIF

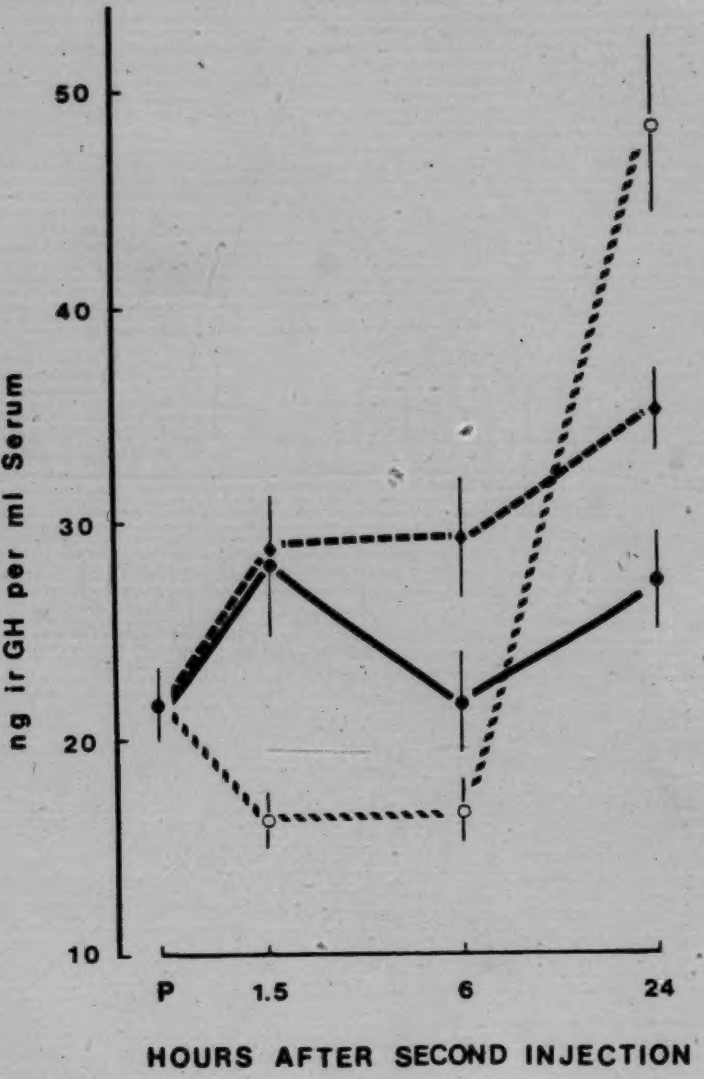

ng ir GH per ml Serum
50
40
30
20
10
P
1.5
6
24
HOURS AFTER SECOND INJECTION

Fig. 2.2. (Experiment 2.2). Changes in serum immunoreactive growth hormone (ir GH) levels in 18 male goldfish before and after two injections of somatostatin (1.0 μg SRIF/g BWt), given 12 hours apart. The post-treatment sample was taken at 13.5 hours after the presample and 1.5 hours after the second SRIF injection (same data as in Fig. 2.1).

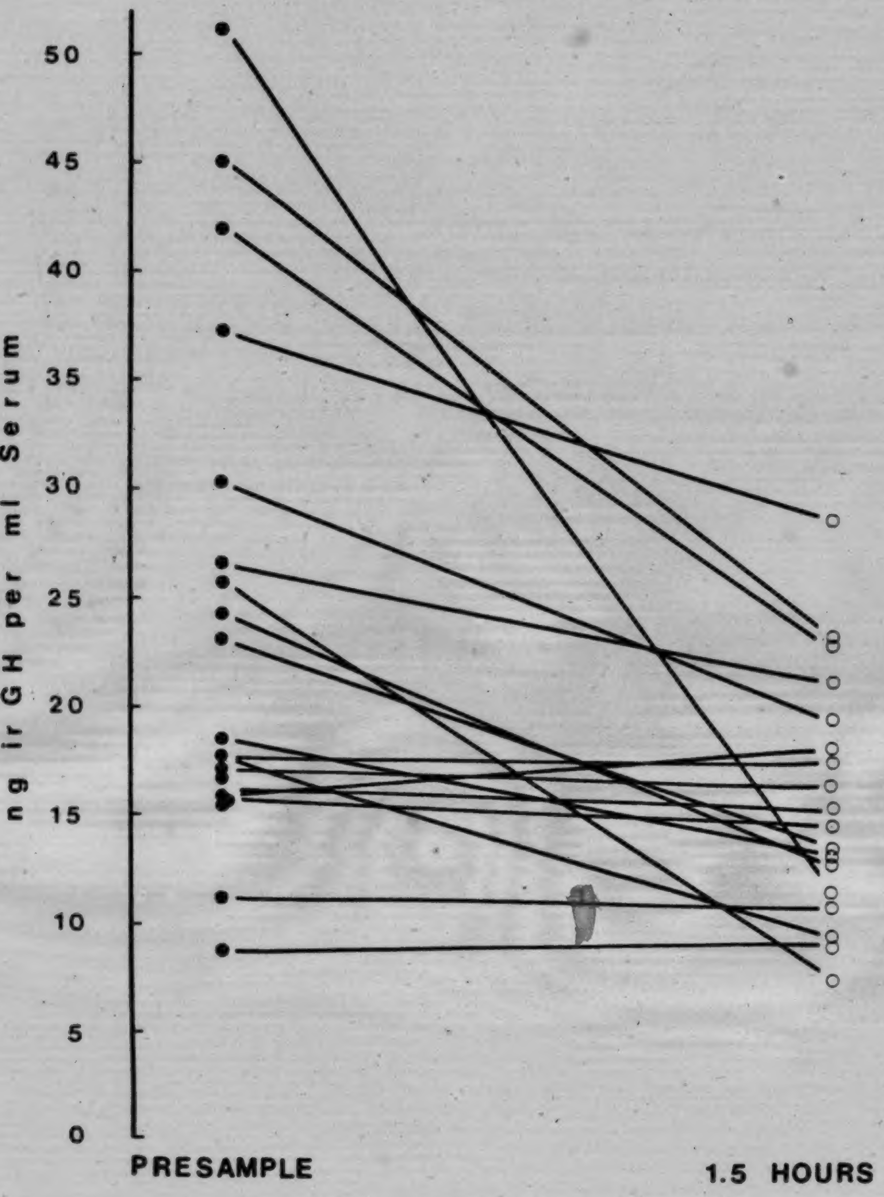

ng ir GH per ml Serum
50
45
40
35
30
25
20
15
10
5
0
PRESAMPLE
1.5 HOURS

Fig. 2.3 shows the results of 2 injections of either 0.1, 0.5 or 1.0 μg SRIF/g BWt given 12 hr apart in sexually mature male goldfish (GSI = 2.96 ± 0.37%, Experiment 2.3). Both 1.0 and 0.5, but not 0.1 μg SRIF/g BWt caused a significant decrease in serum ir GH levels at 1.5 hr following the second injection compared to the presample control mean value. Both the highest and intermediate doses of SRIF also caused a significant ($p < 0.01$) rebound increase in serum ir GH levels at 24 hr following the second injection. Interestingly, although the lowest dose of SRIF (0.1 μg/g BWt) caused a significant decrease in serum ir GH levels compared to the vehicle, but not presample control group at 1.5 hr following the second injection (but see below), this dose was associated with a rebound increase at 6 hr following the second injection (Fig. 2.3). Although a vehicle control group (N=10) was included in Experiment 2.3 (data not shown), the presample serum ir GH value, in addition to the 1.5 and 6 hr values, of this group were significantly greater than those of the 3 SRIF-treated groups, obviating statistical comparison with this group.

Drug Experiments

The effects of NE on serum ir GH levels in female goldfish were different when results of experiments performed between November and March were compared with those of May and June. Tables 2.2, 2.3, 2.4, 2.5 and 2.6 summarize the results of a series of experiments conducted between November and March; Tables 2.7, 2.8 and Fig. 2.4 are from the May and June experiments.

In March, injection of NE (1 and 100 μg/g BWt) caused a

Fig. 2.3. (Experiment 2.3). The effect of three doses of synthetic linear somatostatin (SRIF) on serum immunoreactive growth hormone (ir GH) levels in male goldfish. All fish were sampled immediately prior to the first of two intraperitoneal injections given 12 hours apart (presample, p) and at 1.5, 6 and 24 hours following the second injection. The values shown are $\overline{X} \pm SE$.

-------------- 0.1 μg SRIF/g BWt N=10

—————————— 0.5 μg SRIF/g BWt N=11

////////////// 1.0 μg SRIF/g BWt N=10

Paired t-test using log-transformed data ($p < 0.01$).

0.1 $\underline{1.5 \quad p} \quad \underline{6 \quad 24}$

0.5 $\underline{1.5 \quad \smash{\underline{6}}}\underline{\quad p} \quad 24$

1.0 $1.5 \quad \underline{p \quad 6} \quad 24$

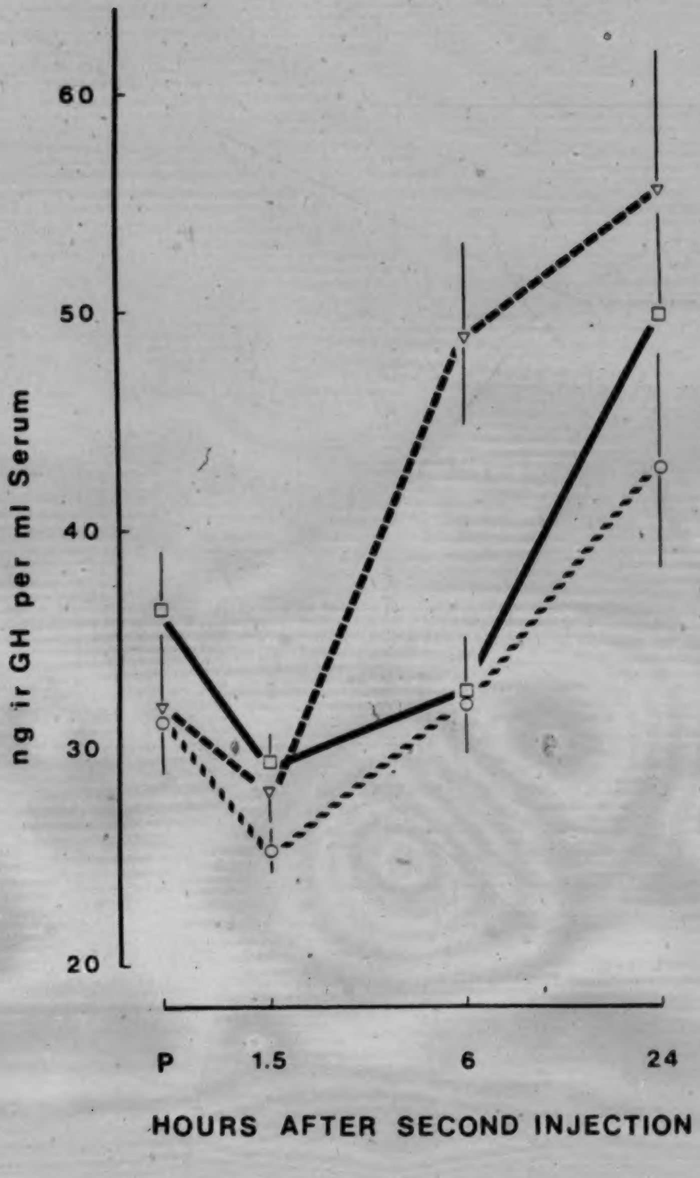
ANOVA and Duncan's multiple range test using log-transformed data ($p < 0.01$).

p $\underline{1.0 \quad 0.1 \quad 0.5}$

1.5 $\underline{1.0 \quad 0.1 \quad 0.5}$

6 $\underline{1.0 \quad 0.5} \quad 0.1$

24 $\underline{1.0 \quad 0.5 \quad 0.1}$


ng ir GH per ml Serum
60
50
40
30
20
P
1.5
6
24
HOURS AFTER SECOND INJECTION

significant increase ($p < 0.05$) in serum ir GH levels at 1 hr post-injection in female goldfish (Table 2.2); however, this effect was not dose-dependent, and the dose of 10 μg NE/g BWt did not cause a significant increase. Although 1 μg NE/g BWt caused a significant increase in serum ir GH levels 1 hr post-injection (Table 2.2), the same dose of NE had no significant effect at 6 hr post-injection in a separate experiment conducted in January (see Table 2.6).

The α-adrenergic agonist clonidine (30 μg/g BWt) resulted in elevated serum ir GH levels at 6 hr post-injection in an experiment in February, 1980 (Table 2.3). In a separate experiment carried out in January, injection of 300 μg clonidine/g BWt resulted in significantly elevated serum ir GH levels compared with the vehicle control group at both 2 and 6 hr post-injection (Table 2.3). Table 2.4 summarizes the results of 3 separate experiments on the effects of a single injection of the catecholamine synthesis inhibitor AMPT on serum ir GH levels in female goldfish. Although a dose of 5 μg AMPT/g BWt resulted in a significant decrease in serum ir GH levels at 6 hr post-injection in November, administration of 100 μg AMPT/g BWt in January had no significant effect. In February 300 μg AMPT/g BWt caused a marked inhibition at 2 hr post-injection in both the mean and range of serum ir GH levels when compared with the vehicle control group (Table 2.4). In one experiment conducted in February, phentolamine (5 μg/g BWt) caused a significant inhibition of serum ir GH levels in female goldfish (Table 2.5); whereas in a separate experiment, also conducted in February, the same dose of propranolol had no significant effect on serum ir GH levels (Table 2.5). Reserpine was without effect at the

TABLE 2.2

Effect of a single intraperitoneal injection of norepinephrine (NE) on serum immunoreactive growth hormone (ir GH) levels 1 hour post-injection in female goldfish acclimated to 12 ± 1°C and a 12L:12D light-dark cycle (11 March, 1980).

treatment	dose (μg/g BWt)	N	ng ir GH per ml serum
vehicle	--	11	18.99 ± 1.62[1]
NE	1.0	10	28.74 ± 2.23
NE	10.0	10	20.77 ± 1.77
NE	100.0	10	33.35 ± 3.92

[1] All data are $\overline{X}$ ± SE.

Results of analysis of variance and Duncan's multiple range test.

V 10 1 100 $p < 0.01$

V 10 1 100 $p < 0.05$

TABLE 2.3

Effect of a single intraperitoneal injection of clonidine on serum immunoreactive growth hormone (ir GH) levels at 2 and 6 hours post-injection in female goldfish acclimated to 12 ± 1°C and a 12L:12D light-dark cycle.

date	treatment	dose (μg/g BWt)	N	ng ir GH per ml serum	
				2 hours post-injection	6 hours post-injection
7 Feb. 1980	vehicle	--	8	52.68 ± 19.80[1]	15.88 ± 2.64
	clonidine	30.0	7	22.90 ± 2.33	23.83 ± 2.75[2]
25 Jan. 1980	vehicle	--	7	16.90 ± 2.27	10.16 ± 1.15
	clonidine	300.0	7	25.36 ± 2.39[2]	22.37 ± 1.61[3]

[1] All data are $\overline{X}$ ± SE.

[2] Significantly different from vehicle control value ($p < 0.05$).

[3] Significantly different from vehicle control value ($p < 0.01$).

TABLE 2.4

Effect of a single intraperitoneal injection of alpha-methyl-paratyrosine (AMPT) on serum immunoreactive growth hormone (ir GH) levels at 2, 6 and 24 hours post-injection (presample taken immediately prior to injection) in female goldfish acclimated to 12 ± 1°C and a 12L:12D light-dark cycle.

date	treatment	dose (µg/g BWt)	N	ng ir GH per ml serum				% change over presample
				hours post-injection				
				0 (presample)	2	6	24	
23 Nov. 1979	vehicle	--	8	12.57±1.24[1]		16.76±1.52	17.39±2.39[2]	
	AMPT	5.0	9	12.01±0.74		11.44±1.12[3]	15.61±1.73	
15 Jan. 1980	vehicle	--	8	30.56±2.10		26.84±4.88		-11.51±19.09
	AMPT	100.0	8	21.30±2.24[2]		26.95±5.14		27.05±21.83
7 Feb. 1980	vehicle	--	8		52.68±19.80			
	AMPT	300.0	8		14.14±2.83[4]			

[1] All data are $\overline{X} \pm SE$.

[2] Significantly different from presample vehicle value ($p < 0.05$).

[3] Significantly different from vehicle control value ($p < 0.02$).

[4] Significantly different from vehicle control value ($p < 0.01$).

TABLE 2.5

Effect of a single intraperitoneal injection of phentolamine or propranolol on serum immunoreactive growth hormone (ir GH) levels at 2 and 6 hours post-injection in female goldfish acclimated to 12 ± 1°C and a 12L:12D light-dark cycle.

date	treatment	dose (μg/g BWt)	N	ng ir GH per ml serum	
				2 hours post-injection	6 hours post-injection
7 Feb. 1980	vehicle	--	8	52.68 ± 19.80[1]	15.88 ± 2.64
	phentolamine	5.0	7	15.76 ± 3.08[2]	26.91 ± 4.63
18 Feb. 1980	vehicle	--	8	7.74 ± 1.71	11.48 ± 2.81
	propranolol	5.0	7	11.09 ± 1.58	14.41 ± 2.57

---

[1] All data are $\overline{X}$ ± SE.

[2] Significantly different from vehicle control value ($p < 0.01$).

TABLE 2.6

Effect of a single intraperitoneal injection of norepinephrine (NE) and reserpine on serum immunoreactive growth hormone (ir GH) levels 6 hours post-injection in female goldfish acclimated to 12 ± 1°C and a 12L:12D light-dark cycle.

date	treatment	dose (µg/g BWt)	N	ng ir GH per ml serum		% change over presample value
				presample	6 hours post-injection	
15 Jan. 1980	vehicle	--	8	30.56 ± 2.10[1]	26.84 ± 4.88	-11.51 ± 19.09
	NE	1.0	8	20.81 ± 2.03[2]	15.28 ± 1.82[3]	-26.31 ± 7.04
	reserpine	1.0	8	25.63 ± 4.68	25.19 ± 3.02	11.81 ± 17.30

[1] All data are $\overline{X}$ ± SE.

[2] Significantly different from vehicle control value ($p < 0.01$).

[3] Significantly different from presample control value ($p < 0.01$).

single dose and sample time employed (see Table 2.6).

In contrast to the results obtained between November and March, both 0.1 and 1 μg NE/g BWt caused significant decreases in serum ir GH levels at 0.5 hr post-injection in female goldfish acclimated to 12 ± 1°C and a 12L:12D light-dark cycle in May (Table 2.7). In a comparable experiment using goldfish acclimated to 12 ± 1°C and a 16L:8D light-dark cycle, both 10 and 100 μg but not 1 μg NE/g BWt caused significant reductions in mean serum ir GH levels at 0.5 hr post-injection in female goldfish (Table 2.8). Fig. 2.4 demonstrates that the significant inhibition of NE on serum ir GH levels in May and June is transient, beginning at 0.5 hr and ending between 1 and 2 hr post-injection; there was no significant difference between vehicle and NE-treated fish at either 2 or 6 hr post-injection.

Tables 2.9 and 2.10 show the results of a single injection of either L-DOPA or DA on serum ir GH levels in female goldfish acclimated to 12 ± 1°C and a 12L:12D light-dark cycle. Except for the 10 μg L-DOPA/g BWt group, all dosages of L-DOPA, from 50 to 200 μg/g BWt, significantly increased ($p < 0.01$) serum ir GH levels in a dose-dependent manner at 1 hr post-injection (Table 2.9). In a separate experiment L-DOPA (100 μg/g BWt) also elevated serum ir GH levels at 6 hr post-injection (Table 2.9). Table 2.10 shows that all three doses of DA tested (1, 10 and 100 μg/g BWt) caused significant increases ($p < 0.01$) in serum ir GH levels at 1 hr post-injection, although there was no evidence of a dose-response pattern. In a separate experiment DA (100 μg/g BWt) had no detectable effect on serum ir GH levels at both 2 and 6 hr post-injection (Table 2.10).

TABLE 2.7

Effect of a single intraperitoneal injection of norepinephrine (NE) on serum immunoreactive growth hormone (ir GH) levels 0.5 hours post-injection in female goldfish acclimated to 12 ± 1°C and a 12L:12D light-dark cycle.

date	treatment	dose (μg/g BWt)	N	ng ir GH per ml serum		% change over presample value
				presample	0.5 hours post-injection	
12 May 1980	vehicle	--	10	28.78 ± 3.00[1]	26.92 ± 3.34	-2.72 ± 9.65
	NE	0.1	11	30.88 ± 2.10	23.07 ± 1.41[2]	-22.28 ± 6.57[3]
	NE	1.0	10	41.63 ± 5.34	23.02 ± 1.40[2]	-38.43 ± 6.34[4]
	NE	10.0	11	27.91 ± 4.22	23.34 ± 1.79	-4.73 ± 9.47

---

[1] All data are $\overline{X}$ ± SE.

[2] Significantly different from presample control value ($p < 0.01$).

[3] Significantly different from vehicle control value ($p < 0.05$).

[4] Significantly different from vehicle control value ($p < 0.01$).

TABLE 2.8

Effect of a single intraperitoneal injection of norepinephrine (NE) on serum immunoreactive growth hormone (ir GH) levels 0.5 hours post-injection in female goldfish acclimated to 12 ± 1°C and a 16L:8D light-dark cycle.

date	treatment	dose (μg/g BWt)	N	ng ir GH per ml serum		% change over presample value
				presample	0.5 hours post-injection	
12 June 1980	vehicle	--	8	17.42 ± 1.71[1]	22.11 ± 1.71[2]	30.06 ± 9.30
	NE	1.0	8	20.41 ± 5.17	29.35 ± 6.78[3]	53.01 ± 18.59
	NE	10.0	7	21.61 ± 2.67	17.85 ± 2.10	-10.70 ± 14.80[4]
	NE	100.0	8	22.73 ± 4.70	12.79 ± 2.64[5]	-45.02 ± 7.08[6]

1 All data are $\bar{X}$ ± SE.

2 Significantly different from presample control value ($p < 0.01$).

3 Significantly different from presample control value ($p < 0.05$).

4 Significantly different from vehicle control value ($p < 0.05$).

5 Significantly different from presample control value ($p < 0.02$).

6 Significantly different from vehicle control value ($p < 0.001$).

Fig. 2.4. The effect of norepinephrine (NE) (------------) and physiological saline (PS) (————————) on serum immunoreactive growth hormone (ir GH) levels in female goldfish acclimated to 12 ± 1^{o}C and a 12L:12D light-dark cycle. The experiment was done in June, 1980. Values shown are the $\overline{X} \pm SE$ of 10 fish at each sample time. The NE-treated fish had significantly (*) reduced ($p < 0.05$) serum ir GH levels at 0.5 and 1 hr post-injection compared to vehicle-control group (Student's t-test). Results of analysis of variance and Duncan's multiple range test ($p < 0.01$) are shown below (groups joined by underline are not significantly different).

PS	0	1	0.5	6	2
NE (100 μg/g BWt)	1	0.5	6	0	2

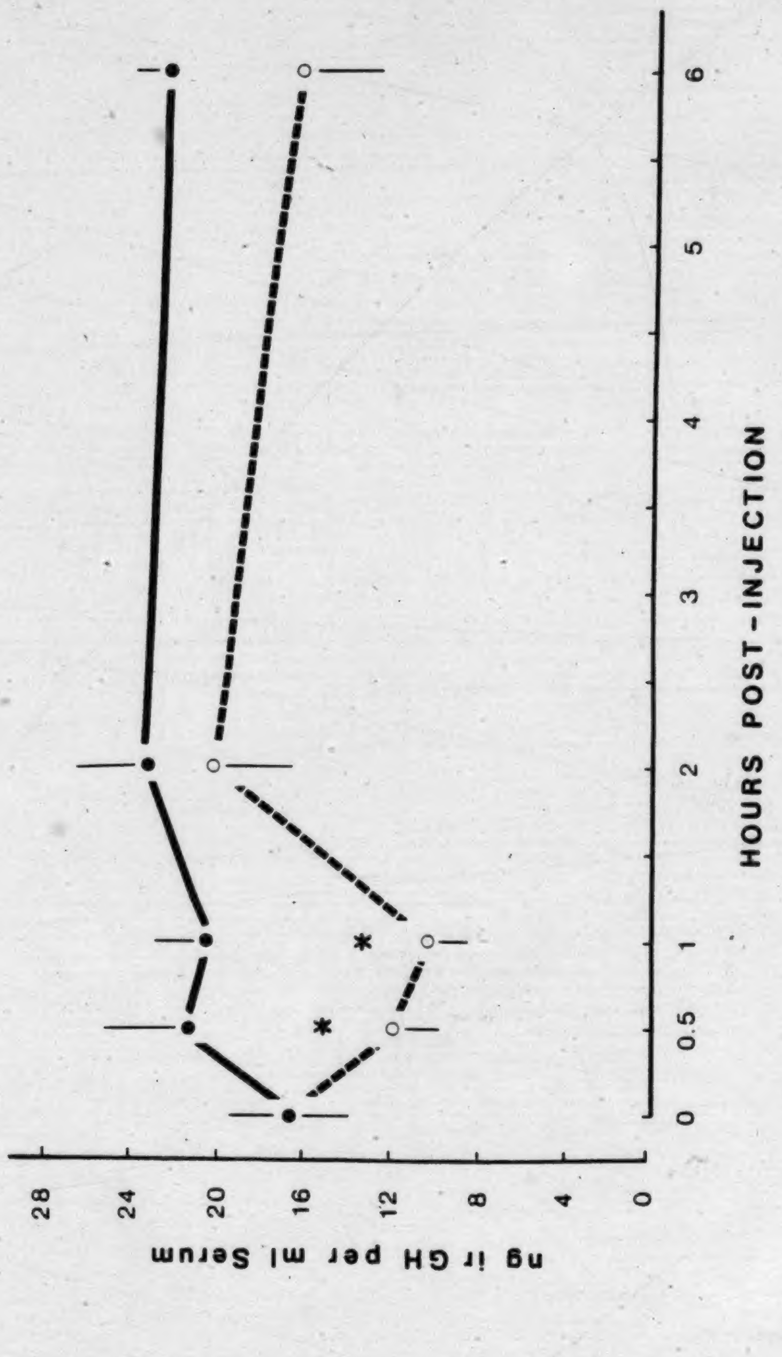

ng ir GH per ml Serum
28
24
20
16
12
8
4
0
HOURS POST-INJECTION
0
0.5
1
2
3
4
5
6
*
*

TABLE 2.9

Effect of a single intraperitoneal injection of dihydroxyphenylalanine (L-DOPA) on serum immunoreactive growth hormone (ir GH) levels 1 and 6 hours post-injection in female goldfish acclimated to 12 ± 1°C and a 12L:12D light-dark cycle.

date	treatment	dose (μg/g BWt)	N	ng ir GH per ml serum	
				1 hour post-injection	6 hours post-injection
10 Apr. 1980	uninjected control	--	5	22.24 ± 2.86^{1}	
	vehicle	--	10	23.61 ± 2.97	
	L-DOPA	10	9	23.30 ± 2.02	
	L-DOPA	50	8	40.45 ± 6.01^{2}	
	L-DOPA	100	5	47.44 ± 5.18^{2}	
	L-DOPA	200	6	56.36 ± 9.84^{2}	
7 Feb. 1980	vehicle	--	8		15.88 ± 2.64
	L-DOPA	100	8		38.32 ± 11.21^{2}

[1] All data are $\overline{X} \pm$ SE.

[2] Significantly different from vehicle control value ($p < 0.01$).

TABLE 2.10

Effect of a single intraperitoneal injection of dopamine (DA) on serum immunoreactive growth hormone (ir GH) levels 1, 2, 6 and 24 hours post-injection in female goldfish acclimated to 12 ± 1°C and a 12L:12D light-dark cycle.

date	treatment	dose (μg/g BWt)	N	ng ir GH per ml serum				
					hours post-injection			
				presample	1	2	6	24
11 June 1980	vehicle	--	11		12.56±2.43[1]			
	DA	1.0	10		22.14±5.48[2]			
	DA	10.0	10		18.05±2.77[2]			
	DA	100.0	10		23.97±5.81[2]			
18 Feb. 1980	vehicle	--	8			7.74±1.71	11.48±2.81	
	DA	100.0	5			16.60±2.09	11.71±2.79	
23 Nov. 1979	vehicle	--	8	12.57±1.24			16.76±1.52	17.39±2.39[3]
	DA	1.0	9	14.46±1.03			15.63±1.10	15.26±2.57

[1] All data are $\overline{X}$ ± SE.

[2] Significantly different from vehicle control value ($p < 0.05$).

[3] Significantly different from presample control value ($p < 0.05$).

In a third experiment completed in November, 1979, 1 μg DA/g BWt had no significant effect on serum ir GH levels at 6 or 24 hr post-injection compared to either the presample or vehicle control value.

Combination Experiment

The results of an experiment concerning the effects of two ip injections (12 hr apart) of combinations of SRIF, L-DOPA and CARBIDOPA are shown in Table 2.11. SRIF (1 μg/g BWt) caused a significant reduction in serum ir GH levels at 1.5 hr following the second injection compared to both the vehicle and simultaneous presample control values (Table 2.11; -62.4 ± 6.3% of presample value for SRIF group compared to 96.7 ± 31.9% for vehicle group, $\overline{X}$ ± SE). Serum ir GH levels rebounded to levels which were not significantly different from both control values by 24 hr following the second injection of SRIF. L-DOPA (50 μg/g BWt) caused a marked increase in serum ir GH levels which was significantly different from the presample value, but not the vehicle-injected group, at both 1.5 and 24 hr post-injection. Injection of a combination of SRIF and L-DOPA alone had no statistically significant effect on serum ir GH levels at either 1.5 or 24 hr post-injection compared to both the presample and vehicle-injected control values. The large SE of the mean serum ir GH level of this group, at 1.5 hr following the second injection, is due to individual fish showing either a marked increase (N=5) or a marked decrease (N=6) in serum ir GH levels relative to the presample control ir GH value. Injection of the peripheral decarboxylase inhibitor CARBIDOPA (50 μg/g BWt) had no significant effect on serum ir GH levels at either 1.5 or 24 hours following the second

TABLE 2.11

Effects of two intraperitoneal injections of physiological saline (vehicle), somatostatin (SRIF), dihydroxyphenylalanine (L-DOPA) and 2-(3,4 dihydroxybenzyl) 2 hydrazinopropionic acid (CARBIDOPA) given 12 hours apart on serum immunoreactive growth hormone (ir GH) levels in male goldfish acclimated to 24°C and a 16L:8D light-dark cycle (17 June, 1981).

			ng ir GH per ml serum		
			presample	hours after second injection	
				1.5	24
treatment	dose (µg/g BWt)	N			
vehicle	--	19	43.20[1] ± 6.92	61.81 ± 7.11	55.52 ± 5.46
SRIF	1	10	59.71 ± 8.49	20.19[2] ± 4.58	68.45 ± 7.67
SRIF+L-DOPA	1,50	11	40.74 ± 4.59	48.00 ± 10.88	65.04 ± 8.04
L-DOPA	50	13	36.07[3] ± 6.00	79.05[4] ± 6.70	57.33[4] ± 7.70
L-DOPA+ CARBIDOPA	50,50	9	46.83 ± 7.50	103.41[2] ± 8.37	41.45 ± 6.94
CARBIDOPA	50	5	52.42 ± 17.14	41.35 ± 9.51	68.46 ± 11.07

[1] All data are $\bar{X}$ ± SE.

[2] Significantly different from both presample and vehicle control values ($p < 0.001$).

[3] Significantly different from SRIF presample control value ($p < 0.05$).

[4] Significantly different from presample control value ($p < 0.001$).

injection. At 1.5 hr following the second injection of a combination of L-DOPA and CARBIDOPA, serum ir GH levels were significantly increased when compared to both the presample and vehicle control values, whereas injection of L-DOPA alone caused a significant increase in serum ir GH levels only in comparison with the presample control value (154.0 ± 44.0 and 105.6 ± 58.0% of presample value for L-DOPA-CARBIDOPA combination and L-DOPA, respectively, $\overline{X} \pm SE$).

DISCUSSION

Previous investigations demonstrated that the presence of immunoreactive SRIF in the brains of teleosts (RIA; Vale *et al.*, 1976; King and Millar, 1979; immunohistochemistry, Dubois *et al.*, 1974, 1978, 1979), and that SRIF is capable of inhibiting GH released from teleost pituitaries cultured *in vitro* as measured by RRA (Fryer *et al.*, 1979). However, without data concerning the effects of SRIF on serum GH levels in intact fish it is impossible to assess the role that this neuropeptide may have in the regulation of GH secretion in teleosts (see INTRODUCTION). The present study has, for the first time, demonstrated an inhibitory action of synthetic mammalian SRIF on serum ir GH levels in intact male goldfish. In four separate experiments two ip injections of 1.0 µg SRIF/g BWt given 12 hr apart caused a significant depression of serum ir GH levels at 1.5 hr following the second injection. In addition, at doses as low as 0.5 µg SRIF/g BWt serum ir GH levels were also lowered at 1.5 but not at 6 hr after injection, suggesting that the serum half-life of SRIF in goldfish is limited. These results are comparable with those of mammals where a number of studies have estimated the half-life of SRIF to be between 2 and 25 min, depending on the species and experimental procedures employed (Yen *et al.*, 1974; Brazeau *et al.*, 1974; Schusdziarra *et al.*, 1979).

Analysis of the changes in serum ir GH levels in individual goldfish treated with SRIF indicates that when pretreatment serum levels are less than about 25 ng/ml, SRIF usually had no detectable inhibitory effect. Furthermore, it is apparent that the higher the initial pretreatment ir GH levels, the greater the inhibitory effect of SRIF (see Fig. 2.2). Although SRIF has been found to inhibit both spontaneous and stimulated secretion of GH in a variety of mammalian *in vitro* systems and to inhibit stimulated GH secretion *in vivo* (for review: McQuillan, 1977, 1980), a number of mammalian studies have found no effect of SRIF on basal serum GH levels (Lovinger *et al.*, 1974; Bryce *et al.*, 1975; Davis, 1975; Harvey *et al.*, 1978). In Chapter 4 results are presented indicating GH is secreted in bursts in goldfish, as in mammals. It is possible then that SRIF may act by inhibiting only the spontaneous secretory bursts and may be without effect on the basal serum GH levels. The only published account of the effect of SRIF on GH secretion in fishes (Fryer *et al.*, 1979) demonstrated the dose-dependent inhibition of GH released from pituitary glands of tilapia, but did not determine if the spontaneous GH secretion occurred in pulses under *in vitro* conditions. It is interesting to speculate that in the goldfish the basal GH levels, in contrast to elevated levels, may depend on a GH releasing factor as postulated by Peter and McKeown (unpublished results; see Chapter 3), although other studies have provided only limited (see Chapter 3) or no data (Fryer, 1981) to support such a hypothalamic factor for the goldfish.

The finding that SRIF is capable of inhibiting the release of GH in a variety of mammals (for reviews: Chiodini and Liuzzi, 1979; MacQuillan, 1980) and from pituitary glands of the domestic fowl (Hall and Chadwick, 1976) and the teleost, *S. mossambicus*

(Fryer *et al.*, 1979) has been obtained using a wide variety of independently developed and validated GH RIAs and RRAs. Thus, the finding that SRIF caused a significant reduction in serum ir GH levels in goldfish at 1.5 hr following the second injection is important for two reasons. First, it confirms and extends the results of Fryer *et al.* (1979) by demonstrating the same effect of SRIF on fish pituitaries which are in the normal communication with the hypothalamus, as opposed to *in vitro* conditions. Secondly, this result implies that the cGH RIA developed in Chapter 1 shares, in common with a wide variety of RIAs and RRAs, the property of measuring the inhibition of GH caused by SRIF in a homologous or near-homologous system.

In male goldfish acclimated to 12 ± 1°C and a 16L:8D light-dark cycle, two ip injections of 1 and 0.5 μg SRIF/g BWt resulted in a very marked post-inhibitory rebound in serum ir GH levels at 24 hr post-injection. In a separate experiment conducted under similar conditions two ip injections of 1 μg SRIF/g BWt also resulted in a significant post-inhibitory rebound in serum ir GH levels at 24 hr post-injection. With the lowest dose of SRIF (0.1 μg/g BWt), the post-inhibition rebound was detectable earlier, by 6 hr post-injection. In the combination experiment (Table 2.11), two ip injections of SRIF (1 μg/g BWt) caused an inhibition of serum ir GH levels at 1.5 hr, but was not associated with a statistically significant rebound effect at 24 hr following the second injection, although the results are suggestive of an increased level at the 24 hr sample. Since the combination experiment was performed after acclimation to 24 ± 2°C as opposed to 12 ± 1°C used for the SRIF experiments and since the control (both presample and vehicle-injected groups) serum ir GH levels were greater in the combination experiment than in the SRIF experiments, it is not possible to directly compare the results of these experiments. Consistent with the post-inhibitory rebound increase in goldfish, other studies have shown that at the end of SRIF infusions plasma GH rebounds to levels exceeding those measured in basal conditions in humans (Hall *et al.*, 1973; Besser *et al.*, 1974), baboons (Ruch *et al.*, 1974) and rats (Martin *et al.*, 1974). The present study has clearly demonstrated the inhibitory action of SRIF on serum ir GH levels in the goldfish and illustrated similarities in the dynamics of the SRIF effect with that obtained in a variety of mammalian and avian species.

Administration of synthetic mammalian TRH (1 μg/g BWt) to male goldfish caused a significant increase in serum ir GH levels at 24 hr, following the second of two injections given 12 hr apart, in comparison with both presample and vehicle-injected control groups. However, there was no detectable increase in serum ir GH levels at either 1.5 or 6 hr after the second TRH injection. If the half-life of TRH in the goldfish is similar to that of mammals (about 5 min, Murad and Haynes, 1980) then the time course of the TRH effect in goldfish would suggest that TRH is not altering serum ir GH levels through a direct action on the pituitary gland. Additional studies and confirmation of the present results are required before TRH can be considered to have a role in the regulation of serum GH levels in goldfish.

The present data indicate that serum ir GH levels in female goldfish can be altered by systemic injections of a variety of catecholamines or drugs which alter the synthesis or action of catecholamines. Results of experiments completed between November and February suggest that NE elevates serum ir GH levels in female goldfish. NE (100 μg/g BWt) and clonidine (30 μg/g BWt), an α-adrenergic receptor stimulant drug, caused marked elevations in serum ir GH levels, whereas the catecholamine biosynthesis inhibitor AMPT (5 μg/g BWt) and the α-adrenergic receptor blocker, phentolamine (5 μg/g BWt) both caused significant decreases in serum ir GH levels. Reserpine (1 μg/g BWt) and the β-adrenergic blocker propranolol (5 μg/g BWt) were not associated with any change in serum ir GH levels. It may be relevant to note that the dose of NE required to elevate serum ir GH levels in goldfish was large in comparison with that previously demonstrated to alter hemodynamics in teleosts (Wood, 1976; Payan and Girard, 1977). However, the finding that much lower doses of clonidine, AMPT and phentolamine caused marked alterations in serum ir GH levels, which corroborates those obtained with NE, favours an action of monoamines on GH secretion as opposed to a nonspecific alteration of GH distribution volume or clearance rate. These results then, are consistent with a stimulatory role for NE in the control of GH secretion in the female goldfish and are in agreement with studies in a variety of experimental mammals where considerable data supports the involvement of α-adrenergic receptors, inside the central nervous system (CNS), involved in the stimulation of GH secretion (for review: Müller *et al.*, 1978; Bluet-Pajot *et al.*, 1980).

In direct contrast to the results of experiments on NE completed in the winter months (see above), experiments conducted in May and

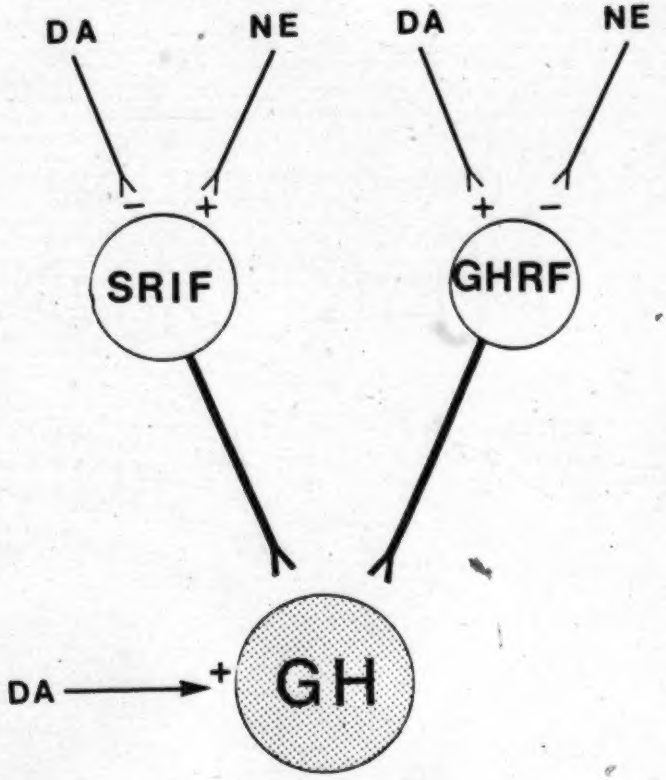
June indicate that at this time of year NE lowers serum ir GH levels in female goldfish. In two separate experiments NE (0.1 and 1 µg/g BWt, Table 2.7; 10 and 100 µg/g BWt, Table 2.8) caused significant decreases in serum ir GH levels in female goldfish. An experiment completed in June, designed to investigate the time course of the serum ir GH response to NE, also demonstrated the inhibitory effect of NE (100 µg/g BWt); decreases in serum ir GH levels were detected at both 0.5 and 1, but not at 2 or 6 hr, post-injection. The results of the present study demonstrate that the changes in serum ir GH levels in response to exogenous administration of NE differ, depending on time of year. If these monoamines exert their effect in female goldfish via a central action, altering the activity of hypophysiotrophic peptidergic neurons (SRIF or GHRF) as suggested by additional results of the present study (see below), then either the goldfish pituitary gland or the hypothalamus is capable of altering the response to the regulatory peptides and/or monoamines, respectively. It may be relevant to note that serum ir GH levels vary seasonally in female goldfish where serum ir GH measurements are much greater during August, at a time when growth rates of many spring spawning fishes are elevated, than in the winter months (see Chapter 4). The serum ir GH response to monoamines may be related to the sexual condition of female goldfish (for review of the goldfish ovarian cycle see: Hontela and Peter, 1978; Cook and Peter, 1980). In the period from November to February, when NE elevated serum ir GH levels, the GSI of female goldfish varied between 3 and 6% (ovarian recrudescence); the GSI of goldfish used in experiments done in June and July when NE lowered serum ir GH levels were between 2 and 3%, reflecting a sexually regressed state of ovarian development. Although the present study has not determined whether the changing serum ir GH response to catecholamines is part of a central mechanism regulating either the annual reproductive cycle or seasonal variations in serum ir GH levels in female goldfish, it is apparent that considerable caution must be exercised in comparing results of neuropharmacological studies in fishes carried out at different times of year.

Systemic injections of both DA (1 μg/g BWt) and L-DOPA (50 μg/g BWt) resulted in significant elevations in serum ir GH levels at 1 hr post-injection in experiments completed between April and June. It is difficult to interpret the increase in serum ir GH levels observed in female goldfish, in response to ip administration of DA, since there was no evidence for a dose-response effect. If DA is unable to penetrate the blood-brain barrier in fishes, as demonstrated for mammals (Weiner, 1980), DA may alter serum ir GH levels in the goldfish by a direct action on the pituitary gland. In contrast to DA, the amino acid precursor of catecholamines, L-DOPA, is able to penetrate the blood-brain barrier in mammals (Bianchine, 1980), and in the present study caused a dose-response effect; increasing doses from 50 μg/g BWt resulted in progressive increases in serum ir GH levels to a maximum 253% increase over control values with 200 μg L-DOPA/g BWt. The serum ir GH response to a combination of L-DOPA and CARBIDOPA was greater than that of an equivalent dose of L-DOPA alone. Furthermore, injection of CARBIDOPA alone had no significant effect on goldfish serum ir GH levels. In mammals, CARBIDOPA is a potent inhibitor of L-DOPA decarboxylation that is unable to penetrate into the CNS; since about 95% of systemically administered L-DOPA is rapidly converted to DA in peripheral tissues (Bianchine, 1980), concurrent administration of both drugs causes an increase in the plasma concentration and half-life of L-DOPA resulting in greater CNS concentrations of L-DOPA (Bianchine and Shaw, 1976). If CARBIDOPA has a similar action in goldfish, it is apparent that results of the present study favour a central action of L-DOPA in elevating serum ir GH levels in goldfish. Since L-DOPA has been shown to exert its main effects by its decarboxylation to DA and/or NE (Bianchine, 1980), and since in the present study both DA and NE have been shown to alter serum ir GH levels, it is likely that the action of L-DOPA in goldfish is mediated by either DA or NE. The finding that NE decreases serum ir GH levels during the summer months in goldfish (see above) at a time when L-DOPA increases serum ir GH levels, suggests that DA may, in part, mediate this increase in response to L-DOPA. In support of this an experiment conducted in June (see Table 2.10) demonstrated that injections of DA resulted in elevated serum ir GH levels.

In addition to demonstrating the inhibitory action of SRIF on serum ir GH levels, the present study has shown that SRIF (1 μg/g BWt) inhibits the L-DOPA induced stimulation of GH secretion in goldfish. The finding that two ip injections of a combination of SRIF and L-DOPA had no effect on serum ir GH levels, whereas injections of SRIF or L-DOPA alone resulted in a significant decrease and increase, respectively, suggests that the action of SRIF and catecholamines are interrelated in the control of GH secretion in the goldfish.

Since L-DOPA elevates serum ir GH levels via a central action in goldfish, probably via decarboxylation to DA or NE (see above), it is apparent that there are a number of possible mechanisms by which monoamines may interact with hypothalamic peptidergic neurons to alter GH secretion. Fig. 2.5 summarizes several possibilities which are all consistent with the data outlined above. Since the results of Chapter 3, together with those of the present study, strongly support a role for SRIF in inhibiting serum GH levels in goldfish it is reasonable to speculate that L-DOPA may stimulate GH secretion by its conversion to DA within the CNS and subsequent inhibition of somatostatinergic neurons. The finding that NE decreased serum ir GH levels in the summer months at the same time of year SRIF was shown to inhibit ir GH levels, suggests that the L-DOPA effect was not mediated by an action of NE on SRIF release. NE could, however, increase the activity of somatostatinergic neurons causing the observed decreases in serum ir GH levels following NE administration in goldfish. Although much work is required to fully elucidate the mechanisms controlling GH release in goldfish, the present study has provided the original information clearly demonstrating the involvement of both catecholamines and SRIF in the regulation of GH secretion in fishes.

Fig. 2.5. Diagrammatic representation of some possible mechanisms involved in the regulation of growth hormone (GH) secretion in the goldfish based on results of experiments done between April and June. Dopaminergic (DA) or noradrenergic (NE) neurons are diagrammed as stimulating (+) or inhibiting (-) hypothalamic peptidergic (somatostatin, SRIF; growth hormone-releasing factor; GHRF) neurons. In turn the SRIF and GHRF neurons could inhibit and stimulate GH release, respectively, by a direct action on the secretory cells of the goldfish pituitary gland. The diagram also indicates that DA could stimulate GH release by a direct stimulatory (+) action on the pituitary gland.


DA
NE
DA
NE
−
+
+
−
SRIF
GHRF
DA
+
GH

Chapter 3. EFFECTS OF HYPOTHALAMIC LESIONS ON SERUM GROWTH HORMONE LEVELS AND GROWTH RATES IN GOLDFISH, *CARASSIUS AURATUS*

INTRODUCTION

In spite of the potential importance of GH in the manipulation of the growth of teleost fishes (for review: Donaldson *et al.*, 1979) there is little known about the control of GH secretion in teleosts (for reviews: Ball *et al.*, 1972; Holmes and Ball, 1974; Peter, 1973; Peter and Fryer, 1981). Experiments involving pituitary transplantation and culture have not provided any clear indication as to either the presence or nature of the neuroendocrine control of GH secretion in fishes. Pituitary autotransplants in the molly, *Poecilia latipinna*, were presumably secreting GH without hypothalamic support, as suggested by the slight growth shown by grafted fish observed over a period from 3 weeks to 8 months (Ball *et al.*, 1972). In addition, somatotropic cells of the eel, *Anguilla anguilla* (Olivereau and Dimovska, 1969; Olivereau, 1970), stickleback, *Gasterosteus aculeatus* (Leatherland, 1970a,b) and *Gambusia sp.* (Chambolle *et al.*, 1981) pituitaries all retain approximately normal appearance after transplantation, although GH cells of *P. formosa* appear less active and fin regeneration was slower than normal after pituitary homotransplantation (Ball *et al.*, 1965; Olivereau and Ball, 1966). Similar to the results obtained using the eel and stickleback, Kayes (1977a) demonstrated that the black bullhead, *Ictalurus melas*, showed only small increments in linear growth after pituitary autotransplantation.

The results of these pituitary transplant studies suggest that GH secretion by the teleost pituitary gland is normally under a tonic level of stimulation by its connections with the hypothalamus, perhaps by a GHRF.

A number of *in vitro* organ culture studies have used PAGE to measure GH (see GENERAL INTRODUCTION) released into the medium by eel pituitary glands (Ingleton *et al.*, 1973; Baker and Ingleton, 1975). Since significant amounts of putative GH were released into the medium for 2 or more weeks in culture, and since histological analysis indicated that the somatotrope cells appeared to be maintained well over this period, it was presumed that the GH cells of the eel pituitary gland were capable of at least some autonomous secretion (Baker and Ingleton, 1975). In contrast to the results obtained using eel pituitaries, when rainbow trout *(Salmo gairdneri)* pituitaries were cultured, putative GH, measured by PAGE and densitometry, was released at a steadily decreasing rate with time in culture, and histological signs of somatotropic hypoactivity were evident as early as 1 week (Baker 1963; Baker 1971 cited in Ball *et al.*, 1972), suggesting that hypothalamic support (e.g. a GHRF) is necessary for the maintenance of GH secretion in the rainbow trout. It is apparent that there are differences in results of pituitary culture experiments involving different teleost species, and between results of pituitary culture and transplant studies. Furthermore, these experiments can not determine whether the pituitary gland in the intact fish is normally under a stimulatory and/or inhibitory influence from the teleost hypothalamus.

More recently Hall and Chadwick (1978) have provided evidence for a GHRF in the eel hypothalamus. Acid extracts of eel hypothalami caused significant increases in the amount of putative GH, as measured by PAGE and densitometry, released into the medium of eel pituitary glands cultured *in vitro* (Hall and Chadwick, 1978). In addition, Hall and Chadwick (1979) demonstrated that hypothalamic extracts from the flounder, *Pleuronectes flesus*, and eel, *A. anguilla*, but not the cod, *Gadus gadus*, increased GH release into the medium from chicken pituitaries incubated *in vitro*. Although these results provide support for a teleost GHRF, it is relevant to note that the PAGE and densitometric technique for GH measurements has not been fully evaluated for use with medium from teleost pituitary gland cultures (see GENERAL INTRODUCTION).

Recently Fryer (1981) indicated that the goldfish *(Carassius auratus)* hypothalamus can influence the somatotrope activity of the pituitary gland. Following electrothermic lesions of the nucleus preopticus (NPO), both light and electron microscopical observations of the pituitary gland indicated enhanced secretory activity of the somatotropes (Fryer, 1981), suggesting that this hypothalamic nucleus was involved in the inhibition of GH release in goldfish, perhaps by a SRIF-like neuropeptide. Lesions of the telencephalon anterior or dorsal to the NPO had no cytological effect on the GH cells. In common with other histophysiological endocrine studies, the correlations between histological or ultrastructural criteria and hormone synthesis, storage or release after lesioning (Fryer, 1981), are speculative without additional information regarding serum and pituitary hormone levels, as well as measurements of the hormone clearance rate (see: Schreibman *et al.*, 1973; McKeown and Peter, 1976; Cook and Peter, 1980). Other studies also suggest that hypothalamic lesions can influence GH secretion in the goldfish. Lesions in the nucleus anterior tuberis (NAT) and large lesions involving most of the nucleus lateralis tuberis (NLT) and NAT caused a significant decrease in serum GH levels as measured by heterologous RIA, suggesting the presence of a GHRF in these brain areas (Peter and McKeown, unpublished results). The finding that lesioning the NPO in goldfish had no significant effect on serum GH levels at 4 weeks post-lesioning (McKeown and Peter, unpublished results) may be considered at variance with a comparable study by Fryer (1981) where similar lesions produced marked alterations in goldfish pituitary ultrastructure at 3 weeks post-operation (see above). While differences between these two studies may be explained on the basis of different sampling times post-lesioning, or the possible presence of daily variations in serum GH levels in goldfish (see Chapter 4), it is apparent that additional lesioning studies are essential to determine what role the hypothalamus may play in the regulation of GH secretion in goldfish. Furthermore, the validity of heterologous RIA measurements of GH used in their study (McKeown and Peter) has been questioned (Nicoll, 1975; see GENERAL INTRODUCTION) and until the RIA is further validated the substance(s) measured is uncertain.

It is clear that there are difficulties in interpreting investigations of the hypothalamic control of GH secretion based on results of pituitary transplant and culture experiments, and that there are difficulties in interpreting results based on GH measurements made with both the heterologous RIA (McKeown and van Overbeeke, 1972) and the PAGE and densitometry methods (Ingleton *et al.*, 1973). In the present study the effects of a variety of hypothalamic radiofrequency lesions on both growth increments and serum ir GH levels were determined to investigate the hypothalamic control of GH secretion in the goldfish. Since the cGH RIA has been fully validated (see Chapter 1) and since changes in BWt and standard length (SL) will be determined concurrently with serum ir GH levels, the present study will provide a major advance over previous investigations of the hypothalamic control of GH secretion in fishes.

MATERIALS AND METHODS

I. General Procedures

The source and general procedures for maintenance and acclimation of goldfish were as described in Chapter 1.

II. Experiments

Experiment 3.1. (July - August, 1977)

In Experiment 3.1, 135 male and female goldfish (25.33 ± 0.39 g BWt, $\overline{X}$ ± SE) were divided evenly between four 96 ℓ flow-through aquaria. The fish were acclimated for 4 weeks to 21 ± 1°C and a 16L:8D light-dark cycle (lights on at 08:00 hr). At the start of the acclimation period all fish were anaesthetized with MS 222 (Sandoz) and individually tagged with metal opercular clips before BWt and length were determined (see below). During the acclimation period, morphometry was recorded weekly to ensure that all fish used in the experiment were growing in a similar manner. Fish showing erratic growth increments or weight losses during this period were removed from experimental aquaria and not included in subsequent data analysis. Fish were fed terramycin-treated Ewos (size 5P) pellets at about 2% of BWt/d, twice daily at approximately 08:00 and 17:00 hr, except on the days of lesioning and blood sampling (see below).

At the end of the acclimation period the fish in each aquarium were divided into three groups: a normal control group, a sham-operated group and a brain-lesioned group. Radiofrequency lesioning and sham operations were performed as described by Peter and Gill

(1975) using size 0 stainless steel insect pins as electrodes. Lesions were made using a voltage of 70 to 85 mV for 30 sec, and the coordinates for electrode placement were as follows: nucleus anterior tuberis (NAT), +0.4, M, D 2.4; nucleus lateralis tuberis (NLT), +0.6, M, D 3.4; nucleus preopticus (NPO), +1.0, M, D 2.2; and for bilateral lesions of the nucleus recessus lateralis (NRL), +0.1, L 0.7, D 2.3 and +0.1, R 0.7, D 2.3. After lesioning or sham-operation, morphometry was measured for 4 weeks as described below. Weekly growth increments in BWt and standard length (SL) were determined for each group according to

$$\frac{BWt_n - BWt_0}{BWt_0} \times 100 \quad \text{and} \quad \frac{SL_n - SL_0}{SL_0} \times 100,$$

where BWt_n and SL_n represent BWt and SL at week n, respectively and BWt_0 and SL_0 represent BWt and SL at the time of lesioning. Thus all the growth increments of the present study (see RESULTS) represent the per cent change in BWt or SL relative to the time of operation (i.e. week 0). Preliminary analysis of changes in BWt or SL during the 4 week duration of Experiment 3.1 showed significant differences between weeks for individual treatment groups, indicating that calculation of average relative instantaneous growth rates as used for the cGH bioassay (see Chapter 1) would be unreliable in estimating the effects of lesioning on growth in this study. Between 11:00 and 13:00 hr on the day of the last weighing and on the two subsequent days, blood samples (about 200 μl) were collected and serum processed for the cGH RIA as described in Chapters 1 and 2. Results of serum ir GH measurements are based on only the first blood sample. Small differences in numbers between serum ir GH and growth measurements for several groups (see RESULTS) were due to the inability to obtain the first blood sample from several fish. After the third blood sample, the fish were killed in excess anaesthetic before the brain, together with the portion of the parasphenoid bone containing the pituitary gland, were dissected and fixed in Bouin's solution. Histological procedures were as described by Peter and McKeown (1974). The placement of lesions was determined histologically and fish were excluded from analyses only if the lesion failed to destroy more than 50% of the particular hypothalamic nucleus. Gonads were also dissected and weighed at the end of the experiment for the determination of gonosomatic index (GSI) (Cook and Peter, 1980). In both Experiments 3.1 and 3.2 the majority (86%) of all the goldfish among the various treatment groups were sexually regressed females (GSI = 2.01 ± 0.17%, $\overline{X}$ ± SE, N=106 for all female fish pooled) and there were never more than 3 males in a single group. The male goldfish included in these experiments were also in a regressed sexual state (GSI = 1.39 ± 0.10%, $\overline{X}$ ± SE, N=19 for all male fish pooled). Since there were no detectable differences between male and female goldfish in either BWt or SL increments or serum ir GH levels regardless of treatment group, data from both sexes were pooled for statistical analyses.

Experiment 3.2. (September - October, 1977)

For Experiment 3.2, 65 goldfish (24.20 ± 0.63 g BWt, $\overline{X}$ ± SE) were placed in a single 293 ℓ flow-through aquarium and acclimated for 4 weeks to 21 ± 1^{o}C and a 12L:12D light-dark cycle (lights on at 08:00 hr). The method and time of BWt and SL measurements and method of feeding were the same as for Experiment 3.1. In addition, fish showing erratic growth increments during the 4 week acclimation period were removed from the experimental aquarium and were not included in the data analysis. At the start of the experimental period the fish were divided among 4 groups: a normal control group, a sham-operated group and two brain-lesioned groups. In this experiment lesion coordinates were +0.04, M, D 2.2 for the NAT and +1.2, M, D 2.1 for the NPP and the voltage used varied between 85 and 105 mV. Other procedures were identical to those described for Experiment 3.1, except that plasma, instead of serum, was collected using the method of Weigand and Peter (1980).

III. Carp Growth Hormone Radioimmunoassay

Details of the cGH RIA are as described in Chapter 1.

IV. Statistical Analyses

Results were analyzed by Student's t-test (Steel and Torrie, 1960).

RESULTS

For Experiment 3.1, the effects of hypothalamic lesions on growth increments are shown in Tables 3.1 to 3.4 and the serum ir GH levels are summarized in Table 3.5. As shown in Table 3.1, lesions in the NAT caused a significant decrease in length increments throughout the 4 week post-lesion period compared to sham controls, but not in comparison to the normal control group. NAT lesions had no significant effect on changes in BWt at any time during Experiment 3.1. Lesions of the NLT resulted in a significant increase in length, but not weight, at 1 week after lesioning, compared with the normal control but not the sham-lesioned control group (Table 3.2), but for weeks 2 to 4 there were no significant differences in either BWt or SL increments among normal control, sham control or NLT-lesioned goldfish. Lesions involving the NPO or bilateral lesions of the NRL had no effects on either weight or length changes during the experiment (see Tables 3.3 and 3.4, respectively). As shown in Table 3.5, lesioning the NAT, NLT, and NPO in Experiment 3.1 had no significant effects on serum ir GH levels. Although bilateral lesions of the NRL in Experiment 3.1 resulted in significantly elevated ($p < 0.01$) serum ir GH compared to the sham control group, the lesion group was not significantly different from the normal control group (statistics not included in Table 3.5).

In contrast to Experiment 3.1, lesioning the NAT had no effect on either BWt or SL increments in Experiment 3.2 (Table 3.6). Lesions involving the NPP resulted in a significantly greater BWt increment

TABLE 3.1

Effects of lesioning the nucleus anterior tuberis (NAT) on body weight (BWt) and standard length (SL) increments in goldfish (Experiment 3.1).

	weeks post-lesion		normal control	sham control	NAT lesioned
		N	26	7	14
% increase over initial SL	1		0.26 ± 0.28^1	3.67 ± 0.69^2	0.71 ± 0.37^3
	2		2.11 ± 0.30^4	4.09 ± 0.94	1.74 ± 0.54^3
	3		4.22 ± 0.42	6.40 ± 1.44	3.60 ± 0.50^3
	4		5.26 ± 0.49^3	8.25 ± 1.73	4.26 ± 0.45^5
% increase over initial BWt	1		1.42 ± 0.64	3.13 ± 1.47	1.52 ± 1.02
	2		9.18 ± 0.83	10.90 ± 2.14	7.50 ± 1.57
	3		12.09 ± 0.98	16.26 ± 4.13	12.62 ± 2.27
	4		17.27 ± 1.31	19.91 ± 5.16	13.57 ± 2.58

[1] All data are $\overline{X} \pm$ SE.

[2] Significantly different compared to normal control value ($p < 0.01$).

[3] Significantly different compared to sham control value ($p < 0.05$).

[4] Significantly different compared to sham control value ($p < 0.02$).

[5] Significantly different compared to sham control value ($p < 0.01$).

TABLE 3.2

Effects of lesioning the nucleus lateralis tuberis (NLT) on body weight (BWt) and standard length (SL) increments in goldfish (Experiment 3.1).

	weeks post-lesion	normal control	sham control	NLT lesioned
	N	26	6	16
% increase in SL	1	0.41 ± 0.31[1]	1.40 ± 0.73	1.42 ± 0.28[2]
	2	2.14 ± 0.30	2.40 ± 1.04	2.85 ± 0.43
	3	4.17 ± 0.41	3.80 ± 1.06	4.55 ± 0.44
	4	5.28 ± 0.47	5.07 ± 1.37	5.33 ± 0.62
% increase in BWt	1	2.21 ± 0.71	1.92 ± 1.99	3.69 ± 0.70
	2	9.21 ± 0.83	5.01 ± 3.07	7.23 ± 0.92
	3	12.73 ± 0.98	11.29 ± 3.24	15.98 ± 1.28
	4	16.89 ± 1.29	11.95 ± 4.41	17.38 ± 1.96

[1] All data are $\overline{X}$ ± SE.

[2] Significantly different compared to normal control value ($p < 0.05$).

TABLE 3.3

Effects of lesioning the nucleus preopticus (NPO) on body weight (BWt) and standard length (SL) increments in goldfish (Experiment 3.1).

	weeks post-lesion		normal control	sham control	NPO lesioned
		N	27	6	15
% increase over initial SL	1		0.40 ± 0.28[1,2]	1.22 ± 0.13	1.14 ± 0.32
	2		1.96 ± 0.34	2.45 ± 0.40	2.73 ± 0.67
	3		4.32 ± 0.46	4.07 ± 0.65	4.16 ± 0.83
	4		5.46 ± 0.51	5.47 ± 0.60	5.80 ± 0.91
% increase over initial BWt	1		1.64 ± 0.73	2.66 ± 1.36	3.34 ± 1.21
	2		8.13 ± 1.08	2.44 ± 1.98[3]	4.82 ± 1.29
	3		11.33 ± 1.14	7.99 ± 2.46	8.64 ± 1.90
	4		16.56 ± 1.38	12.64 ± 3.14	13.27 ± 2.17

[1] All data are $\overline{X}$ ± SE.

[2] Significantly different compared to sham control group ($p < 0.02$).

[3] Significantly different compared to normal control group ($p < 0.05$).

TABLE 3.4

Effects of bilateral lesioning of the nucleus recessus lateralis (NRL) on body weight (BWt) and standard length (SL) increments in goldfish (Experiment 3.1).

	weeks post-lesion	normal control	sham control	NRL lesioned
	N	27	4	11
% increase over initial SL	1	0.46 ± 0.29[1]	1.07 ± 0.87	1.03 ± 0.69
	2	2.69 ± 0.39	3.29 ± 0.96	3.16 ± 0.49
	3	4.74 ± 0.43	4.69 ± 1.12	4.50 ± 0.47
	4	5.99 ± 0.51	6.65 ± 2.40	6.46 ± 0.65
% increase over initial BWt	1	2.21 ± 0.67	2.94 ± 0.84	3.01 ± 0.56
	2	9.66 ± 0.83	5.44 ± 1.73	8.58 ± 1.12
	3	12.74 ± 0.97	9.58 ± 4.44	13.15 ± 1.72
	4	17.82 ± 1.28	12.79 ± 6.97	17.49 ± 2.54

[1] All data are $\overline{X}$ ± SE.

TABLE 3.5

Effects of lesions in the nucleus anterior tuberis (NAT), nucleus preopticus (NPO), nucleus lateralis tuberis (NLT) and bilateral lesions of the nucleus recessus lateralis (NRL) region on serum immunoreactive growth hormone (ir GH) levels in goldfish at 28 days post-lesioning (Experiment 3.1).

		ng ir GH per ml serum		
lesioned area		normal control	sham control	lesioned
	N	6	7	13
NAT	$\overline{X}$	18.70[1]	17.10	18.67
		±	±	±
	SE	1.05	0.95	0.98
	N	7	6	15
NPO	$\overline{X}$	20.06	22.26	25.07
		±	±	±
	SE	1.44	1.86	4.15
	N	6	6	16
NLT	$\overline{X}$	21.51	21.86	23.70
		±	±	±
	SE	1.02	1.62	1.39
	N	7	4	11
NRL	$\overline{X}$	20.69	11.89	21.46
		±	±	±
	SE	1.16	0.86	3.11

[1] All data are $\overline{X}$ ± SE.

TABLE 3.6

Effects of lesioning the nucleus anterior tuberis (NAT) on body weight (BWt) and standard length (SL) increments in goldfish (Experiment 3.2).

	weeks post-lesion	normal control	sham control	NAT lesioned
	N	15	10	10
% increase over initial SL	1	4.09 ± 0.43[1]	3.98 ± 0.87	3.79 ± 0.89
	2	5.92 ± 0.40	5.53 ± 0.30	5.68 ± 1.10
	3	8.42 ± 1.06	6.61 ± 0.72	7.58 ± 0.98
	4	9.88 ± 0.77	7.60 ± 0.91	9.34 ± 1.62
% increase over initial BWt	1	2.54 ± 0.78	0.74 ± 0.40	1.57 ± 1.23
	2	9.93 ± 0.74	6.84 ± 1.69	10.40 ± 2.71
	3	17.79 ± 0.82	12.76 ± 2.95	18.08 ± 3.93
	4	22.13 ± 0.98	16.02 ± 3.68	19.16 ± 1.64

[1] All data are $\overline{X}$ ± SE.

at 4 weeks post-lesioning, compared to both normal and sham control groups (Table 3.7). Furthermore, BWt increments of the NPP-lesioned group were also greater than those of both the sham and normal control groups at all 4 weeks of Experiment 3.2, although the differences were not statistically significant until week 4. At 4 weeks postoperatively the NPP lesions also caused a significant increase in the SL increment compared to the sham control group, and tended to a significant increase in comparison with the normal control group ($t = 1.67$, 21 df, $p < 0.10$; Table 3.7). It is of interest to note that the increases in BWt and SL increments in the NPP-lesioned group were significant (Table 3.7, Experiment 3.2) in spite of the fact that the growth of the normal control group in Experiment 3.2 was significantly greater than that of Experiment 3.1. In Experiment 3.2 there were no significant effects on serum ir GH levels compared to the normal or sham control groups as a result of lesioning the NAT (Table 3.8). However, lesions involving the NPP resulted in a marked increase in serum ir GH levels compared to both the normal and sham control groups at 4 weeks post-lesioning (Table 3.8). At week 4, analysis of BWt increments and serum ir GH levels of individual fish for the normal and sham control groups and the NPP-lesioned group revealed a positive correlation between the two; with increasing serum ir GH levels, the growth increment increased (Fig. 3.1).

Fig. 3.2-1 shows a representative cross-section through the forebrain of a sham-operated goldfish for comparison with a section at about the same level from the forebrain of a fish with a midline

TABLE 3.7

Effects of lesioning the nucleus preopticus periventricularis (NPP) on body weight (BWt) and standard length (SL) incfements in goldfish (Experiment 3.2).

	weeks post-lesion	normal control	sham control	NPP lesioned
	N	15	12	9
% increase over initial SL	1	4.09 ± 0.43[1]	2.76 ± 0.63	3.59 ± 0.56
	2	5.92 ± 0.40	4.79 ± 0.57	5.47 ± 0.51
	3	8.42 ± 1.06	7.34 ± 0.75	8.38 ± 0.71
	4	9.88 ± 0.77	9.02 ± 0.84	11.62 ± 0.70[2]
% increase over initial BWt	1	2.54 ± 0.78	1.58 ± 0.53	2.82 ± 0.63
	2	9.93 ± 0.74	8.85 ± 0.85	10.18 ± 0.81
	3	17.79 ± 0.82	17.00 ± 0.62	19.96 ± 1.37
	4	22.13 ± 0.98	23.15 ± 0.50	28.35 ± 1.71[3]

[1] All data are $\overline{X}$ ± SE.

[2] Significantly different compared to sham control value ($p < 0.05$).

[3] Significantly different compared to normal control ($p < 0.01$) and sham control ($p < 0.02$) values.

TABLE 3.8

Effects of radiofrequency lesions in the nucleus anterior tuberis (NAT) and the nucleus preopticus periventricularis (NPP) on serum immunoreactive growth hormone (ir GH) levels in goldfish at 28 days post-lesioning (Experiment 3.2).

		ng ir GH per ml serum		
lesioned area		normal control	sham control	lesioned
NAT	N	15	10	6
	$\overline{X}$ ± SE	27.29 ± 3.51	27.05 ± 4.18	24.85 ± 2.76
NPP	N	15	12	9
	$\overline{X}$ ± SE	27.29 ± 3.51	22.50 ± 1.51	40.23[1] ± 3.89

[1] Significantly different compared to both normal and sham control values ($p < 0.01$).

Fig. 3.1. Regression of per cent increase over preoperative body weight (BWt) on serum immunoreactive growth hormone levels (ng ir GH per ml serum) at 4 weeks post-lesioning. The solid circles, solid triangles and open diamonds represent the nucleus preopticus periventricularis (NPP) lesioned group (N=9), the sham NPP-lesioned group (N=12) and the normal controls (N=15), respectively.

Per cent increase in BWt = Y; ng ir GH per ml serum = X; p = significance of difference of regression coefficient from zero; N = number of goldfish, r = 0.34.

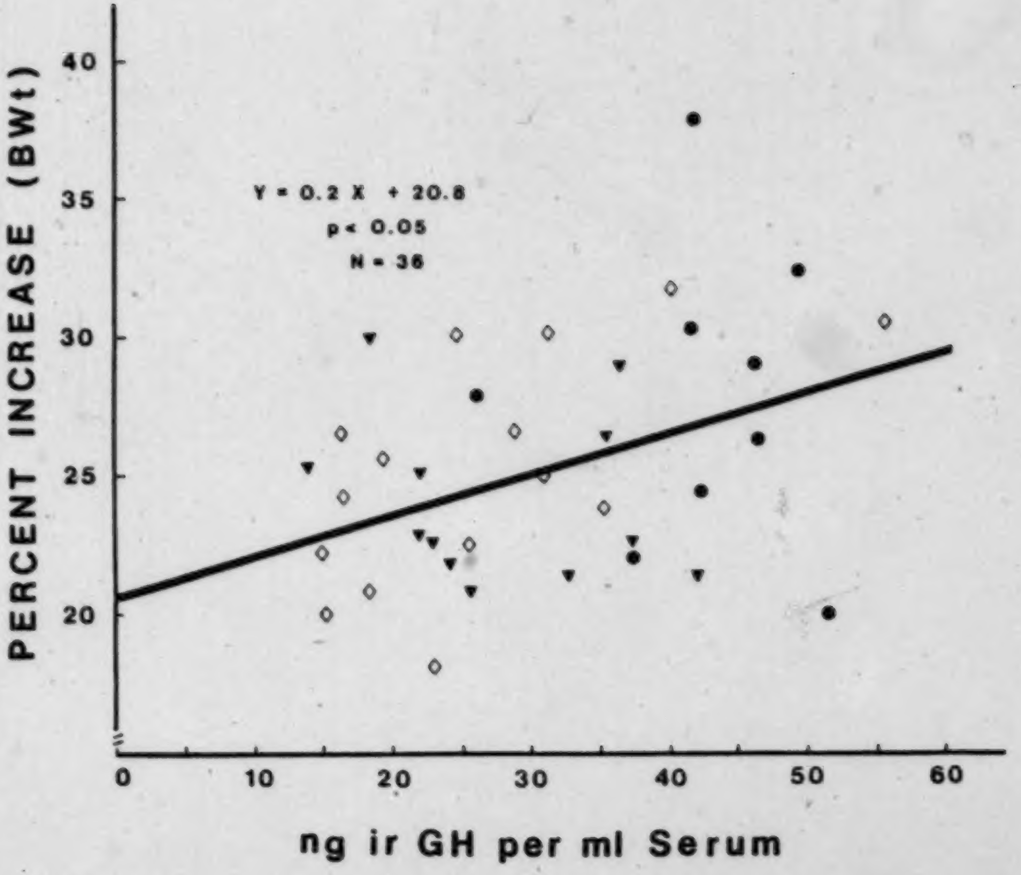
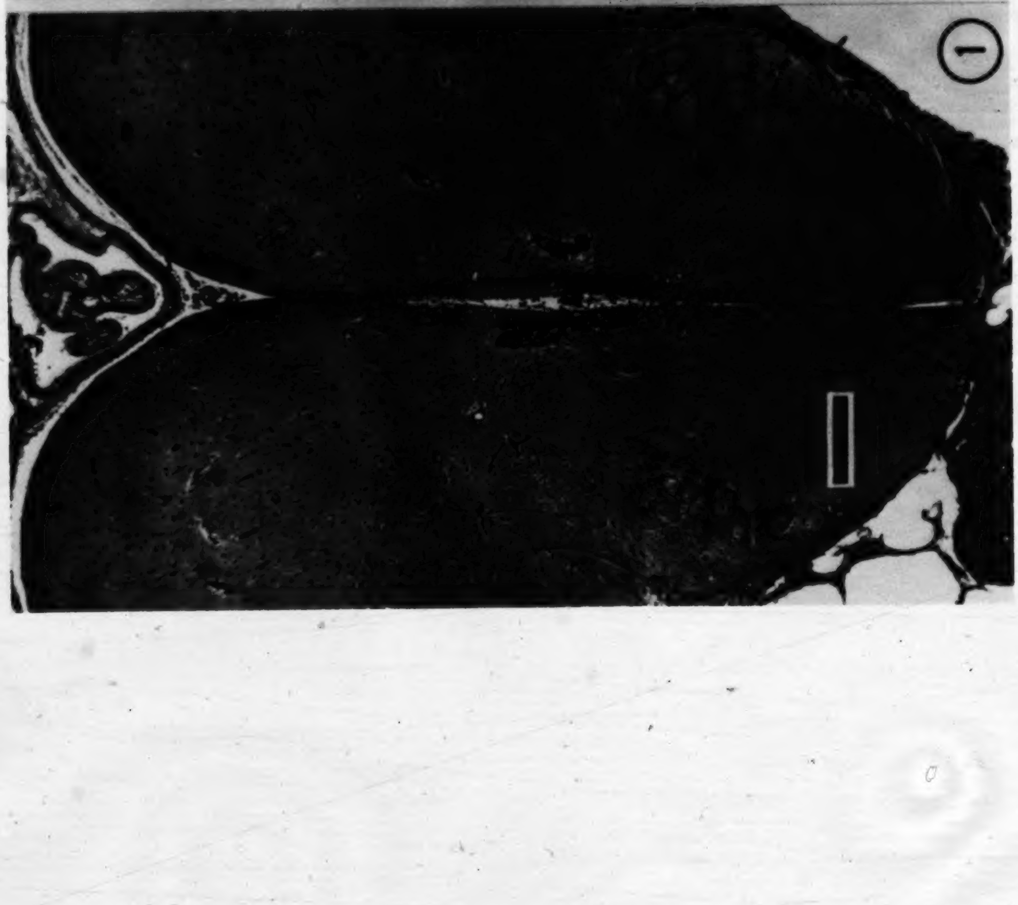

Y = 0.2 X + 20.8
p< 0.05
N = 36
PERCENT INCREASE (BWt)
40
35
30
25
20
0
10
20
30
40
50
60
ng ir GH per ml Serum

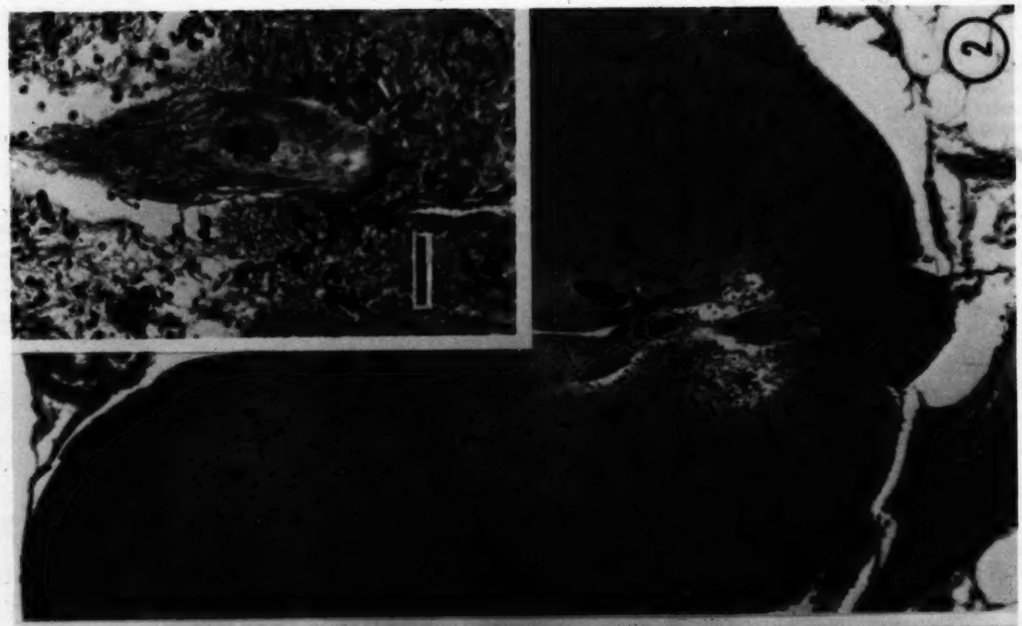
Fig. 3.2-1. Representative cross-section through the forebrain of a sham-operated goldfish at 4 weeks post-operative. The horizontal bar represents 150 μm.

Fig. 3.2-2. Representative cross-section through the forebrain of a goldfish with a midline radiofrequency lesion (arrows) of the nucleus preopticus periventricularis at 4 weeks post-operative. The horizontal bar represents 150 μm.

Fig. 3.2-3. Higher magnification of lesioned area. Note extensive periventricular neural degeneration with necrotic debris. The horizontal bar represents 40 μm.



①

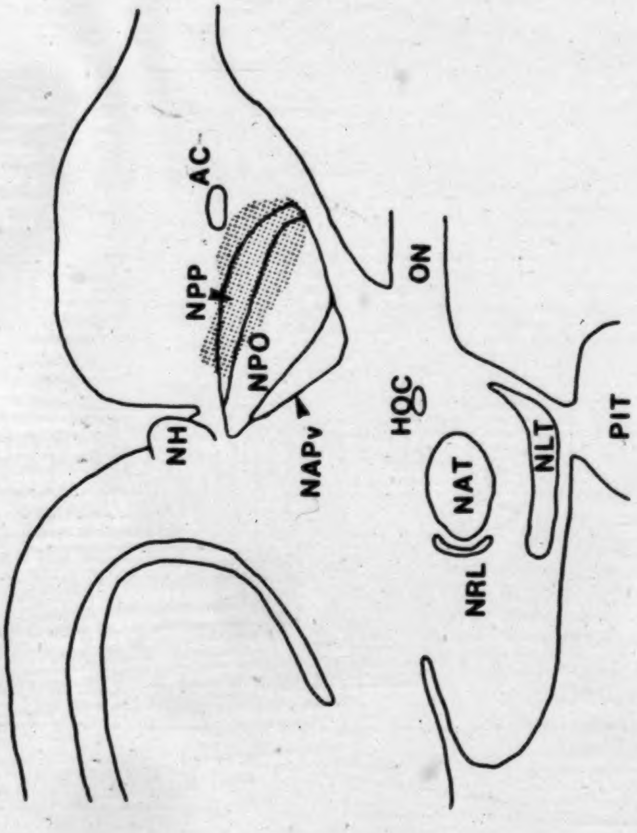


②

lesion (arrows) of the NPP (Fig. 3.2-2). At 4 weeks post-operation the lesioned area of the hypothalamus showed histological evidence of coagulation necrosis (Fig. 3.2-3), including extensive replacement of neural tissue by connective tissue. There was no evidence of degeneration in brain areas other than at the site of lesioning, regardless of the hypothalamic coordinates used in the present study. Fig. 3.3 is a summary diagram of lesions of the individual fish included in the NPP-lesioned group. Of the 9 fish included in this group there was virtually complete destruction of the entire NPP. In addition, in 4 fish the lesion extended in a posterior and posterior-ventral direction to damage the NPO and part of the nucleus anterioris periventricularis (NAPv) (Fig. 3.3).

Fig. 3.3. A diagram of a parasagittal section of the goldfish forebrain (approximately 150 μm lateral to the midline) showing the common area of destruction caused by radiofrequency lesions located primarily in the nucleus preopticus periventricularis of fish in Experiment 3.2. The drawing is magnified approximately 30 X. Terminology is as described by Peter and Gill (1975).

AC, anterior commissure; HOC, horizontal commissure; NAPv, nucleus anterioris periventricularis; NAT, nucleus anterior tuberis; NH, nucleus habenularis; NLT, nucleus lateralis tuberis; NPO, nucleus preopticus; NPP, nucleus preoptic periventricularis; NRL, nucleus recussus lateralis; ON, optic nerve; PIT, pituitary gland.


AC
NPP
NPO
ON
NAPv
HOC
NH
NAT
NLT
PIT
NRL

DISCUSSION

The present study demonstrates that destruction of the NPP in goldfish results in an increase in both growth, as indicated by changes in BWt and SL increments, and serum ir GH levels at 4 weeks after lesioning. The difference in BWt increments between NPP-lesioned fish and both sham and normal control groups increased with time after lesioning, although the effect was not statistically significant until the fourth week. These results suggest the involvement of the NPP in inhibiting GH secretion in goldfish. In support of the present results Fryer (1981) showed that at 14 days after the placement of large lesions in the NPO and much of the NPP, there was a marked increase in nuclear area of the presumed GH cells in the goldfish pituitary. At 21 days after lesioning the NPO there was a marked reduction in the number of both type A neurosecretory fibres innervating GH cells and hormone granules; in addition a proliferation of rough endoplasmic reticulum (RER) was evident, suggesting increased GH secretion (Fryer, 1981). In his study, bilateral lesions of the NPO were 900 μm in length, 400 μm in width and completely destroyed the NPO and NPP with the exception of a few cells of the NPO posteriorly and of the NPP anteriorly (Fryer, 1981). Since in the present study lesions of the NPP, but not the NPO, were effective in increasing growth and ir GH levels, it is likely the apparent enhancement of GH secretion after combined NPO/NPP lesions suggested by cytological and ultrastructural evidence (Fryer, 1981) was primarily due to destruction of only the NPP.

In contrast to lesioning the NPP, lesions of the NAT in female goldfish resulted in significant decreases in growth in comparison with sham-operated fish, as indicated by changes in SL increments (Experiment 3.1). These results suggest that the NAT may play a stimulatory role in GH secretion in goldfish. While the inhibition of SL increments, relative to the sham control group, was evident at all 4 weeks of the post-operative period in the NAT-lesioned fish in Experiment 3.1, these decreases were not significantly different from the normal control group. Although BWt increments of NAT-lesioned fish were not significantly different compared to either control groups, it is apparent that the BWt increments follow a similar pattern to the SL increments of this treatment group (see Table 3.1). Peter and McKeown (unpublished results) have also provided evidence that the NAT is the origin of, or involved with the secretion of some factor that stimulates GH secretion in goldfish. Lesions in the NAT region and in the combined NAT-NLT-NPP region caused decreased serum ir GH levels (Peter and McKeown, unpublished results) measured using the heterologous GH RIA developed by McKeown and van Overbeeke (1972) (also see GENERAL INTRODUCTION). In the present study, serum ir GH levels in NAT-lesioned fish were not significantly different from the control groups in either Experiment 3.1 or 3.2, in spite of the effects on SL increments (see above). However, since significant daily and hourly fluctuations in serum ir GH levels have been demonstrated in goldfish (see Chapter 4) and since blood samples were collected at a single time of day in these studies, it is not possible to rule out an effect of NAT lesions on serum ir GH levels in goldfish. Further investigations are required to determine the temporal sequence of serum ir GH changes, if any, following NAT lesions in goldfish. In summary, the results of NAT lesioning in goldfish in the present study and that of Peter and McKeown (unpublished results) in conjunction with earlier pituitary transplant and culture studies (see INTRODUCTION), provide indirect support for a GHRF in teleosts.

In Experiment 3.2 lesioning the NAT did not result in significant effects on either BWt or SL increments at any of the sample times after lesioning. Since the experimental procedures used in these 2 experiments were very similar (see MATERIALS AND METHODS) it is possible that the different growth response to NAT lesions in goldfish between Experiment 3.1 (July to August) and Experiment 3.2 (September to October) is related to the time of year. While it is premature to postulate a mechanism for the contrasting effects of NAT lesions at the two different times of year, such differences could exist since the changes in serum ir GH response to exogenously administered monoamines have been shown to differ at different times of year (see Chapter 2) and serum ir GH levels in female goldfish exhibit marked variations depending on both temperature and time of year or photoperiod (see Chapter 4).

An important finding of Experiment 3.2 was the relation between serum ir GH levels and BWt increments of normal control and NPP and sham NPP-lesioned fish at 4 weeks post-operative. When data from these groups were combined there was a significant dependence of serum ir GH levels on BWt increments; serum ir GH levels were higher in fish that had greater BWt increments (see Fig. 3.1). Since marked variations in serum ir GH levels can occur in less than 0.5 hr in goldfish (see Chapter 4) and since the collection of blood samples in the present study was performed over a 2 hr period, it is likely that the pattern of serum ir GH levels from individual fish included in Fig. 3.1 was altered by lesioning the NPP, resulting in either a tonic elevation in serum ir GH levels and/or an increased frequency of GH secretory bursts. The finding that greater BWt increments were related to serum ir GH levels suggests that the cGH RIA developed and validated in Chapter 1 measures circulating GH in goldfish that is, at least in part, responsible for growth in goldfish. In support of this, preliminary data presented in Chapter 1 indicated that the rabbit anti-cGH serum used in the RIA cross-reacts with endogenous, circulating biologically active GH in goldfish, causing a slowing of growth.

The results of lesioning experiments and GH secretion in goldfish assume greater importance in view of the recent demonstrations of SRIF in the hypothalamus of teleost fishes (for review: Crim *et al.*, 1978). SRIF has been measured by RIA in whole brain extracts of hagfish and catfish (Vale *et al.*, 1976) and tilipia (King and Millar, 1979), and has been identified in the hypothalamo-hypophysial complex of rainbow trout (*Salmo gairdneri*) with an immunofluorescence technique (Dubois *et al.*, 1974, 1978). More recently, Dubois *et al.* (1979) investigated the anatomical distribution of SRIF in the brain of rainbow trout, and found immunoreactive perikarya in three hypothalamic nuclei, including the NPP, NLT and an unnamed dorsomedial hypothalamic nucleus. Notably, the staining appeared to be more intense and there were more reactive cells in the NPP than in the other two locations (Dubois *et al.*, 1979). In addition, these authors noted that SRIF immunoreactivity in the NPP was quite apart from the NPO, and that in some fish nearly all the NPP perikarya showed a very strong immunofluorescent reaction. Results of hypothalamic lesioning in goldfish in the present study and those of Fryer (1981) are consistent with the studies of the distribution of SRIF immunoreactivity in rainbow trout (Dubois *et al.*, 1978, 1979) inasmuch as the hypothalamic areas noted for SRIF immunoreactivity were also the regions, which when lesioned, resulted in increases in either growth rates and serum ir GH levels,or histological and ultrastructural evidence of increased pituitary GH secretion.

It is relevant to note that SRIF detected in the hypothalamus of tilapia (*Sarotherodon mossambicus*) is probably immunologically identical to synthetic mammalian SRIF and that SRIF extracted from hypothalami of tilapia chromatographs similar to that of several vertebrate species (King and Millar, 1979). Recently, Fryer *et al.* (1979) showed that SRIF inhibited GH, as measured by RRA, released from tilapia pituitary glands maintained in an *in vitro* culture system. *In vivo* studies in the goldfish have also shown that synthetic SRIF can inhibit serum ir GH levels in a dose-dependent manner (see Chapter 2). Assuming that the distribution of SRIF in the brain of goldfish, if present, is similar to that of the rainbow trout (Dubois *et al.*, 1979; and see above), then the results of the present study suggest that the increase in growth detected in goldfish after lesioning the NPP is due to destruction of somatostatinergic neurons in this nucleus and the resulting increase in circulating GH levels.

Chapter 4. DAILY AND SEASONAL VARIATIONS IN SERUM IMMUNOREACTIVE GROWTH HORMONE LEVELS IN THE GOLDFISH, *CARASSIUS AURATUS*

INTRODUCTION

Soon after RIAs for mammalian anterior pituitary hormones came into routine use, it was found by frequent sampling that blood levels of pituitary hormones often fluctuated abruptly (Quabbe *et al.*, 1966; Gallagher *et al.*, 1973). For example, GH secretion in the male rat is characterized by secretory episodes containing 1 to 3 rapid-onset pulses separated by 1 to 2 hr periods of basal serum GH levels (Willoughby and Martin, 1976). Other studies have demonstrated that this ultradian rhythm occurs with a 3.3 hr periodicity and is entrained to the light-dark cycle (Tannenbaum and Martin, 1976). Although GH secretory dynamics have been studied extensively in the rat and rapidly fluctuating serum GH levels have also been described in a variety of experimental mammals (for review: Chiodini and Liuzzi, 1979) and man (Plotnick *et al.*, 1975), few studies have investigated the possible importance of serum GH patterns in either nutrient homeostasis or overall somatic growth (Tannenbaum *et al.*, 1979; Tannenbaum, 1981).

While it is certain that many temperate-zone spring-spawning teleost fishes have seasonal variations in growth rates (for review: Carlander, 1969; Bond, 1979) there are few detailed studies describing the changes in growth rates which occur within a single year. In mature carp, *Cyprinus carpio*, a Cypriniforme closely related to the goldfish, most of the annual growth increment occurs in the late spring and early summer, following the spawning period in early spring (Kawamota *et al.*, 1957). This general pattern of seasonal growth described for the carp is similar to that of a wide variety of spring-spawning teleost fishes including white sucker (*Catastomus commersoni*) (Basset, 1975), barbel (*Barbus barbus*) (Hunt and Jones, 1975), perch (*Perca fluviatilus*) (Le Cren, 1951) and the bluegill sunfish (*Lepomis macrochirus*) (Gerking, 1966). It is likely that temperature, photoperiod, food availability and the metabolic demands that gonadal maturation makes on resources all contribute to these seasonal growth patterns (for reviews: Shul'man, 1974; Brett, 1979; Peter, 1979)..

Although associations have been made between environmental factors and seasonal growth rates, relatively few studies have investigated the possible role of pituitary GH in seasonal growth in fishes. Swift and Pickford (1965) used hypox *Fundulus heteroclitis* to bioassay the GH content of pituitary glands obtained from perch sampled during an annual cycle and found that growth-promoting activity in the pituitary was highest in June, one month prior to the period of maximum growth (Swift and Pickford, 1965). These authors hypothesized that rising water temperature and/or increasing day length stimulates GH synthesis, and that elevated temperature alters GH secretion and target-tissue responsiveness.

Kaul and Vollrath (1974) have studied the annual changes in the presumptive pituitary GH cells of the goldfish (*C. auratus*) by electron microscopy. They found that from October to January the GH cells had relatively little RER and secretory granules, whereas in February and March the GH cells had extensive RER and an increased number of secretory granules. In the summer months the amount of RER in the GH cells was increased further, but the secretory granules were decreased in number compared to the spring months, which suggests that increased rates of GH synthesis and secretion are associated with the summer period of increased growth in the goldfish. It is apparent, however, that without concomitant serum GH measurements the results of Kaul and Vollrath (1974), and also those of Swift and Pickford (1965), are open to alternative explanations. The studies cited above and others (Pickford, 1959; Saunders and Henderson, 1970; Komourdjian *et al.*, 1976, 1977; Adelman, 1977) all suggest from indirect evidence, that endogenous teleost GH is involved in the annual growth cycle. However, without a validated assay suitable for the measurement of endogenous serum GH levels, it has not been possible to assess the possible relationship between annual growth cycles and circulating GH levels in teleost fishes.

In fishes in general, and the goldfish in particular, circadian rhythms appear well established for circulating levels of several teleost hormones (gonadotropin, Hontela and Peter, 1978; thyroxine, Spieler and Noeske, 1978; prolactin, McKeown and Peter, 1976; cortisol, Peter *et al.*, 1978). However, data concerning the possible existence of a circadian rhythm in circulating levels of GH in fishes, based on the single study of kokanee salmon (Leatherland *et al.*, 1974) are equivocal. These authors provided no data concerning reproducibility or stability of the two serum 'GH' peaks and the validity of the heterologous GH RIA used in this study has been questioned (see GENERAL INTRODUCTION).

The present study was designed to determine if goldfish serum ir GH levels, measured with the cGH RIA validated in Chapter 1, might vary in either a daily and/or hourly time period. In addition, the present study describes variations in serum ir GH measurements obtained from large numbers of female goldfish sampled at three separate times of year.

MATERIALS AND METHODS

I. Source and Maintenance of Experimental Animals

The source and general holding procedures for the goldfish used in the experiments of the present study have been described (see Chapter 2).

II. Experiments

Experiments 4.1 and 4.2

At the start of these experiments groups of 7 to 11 female goldfish were fin-clipped for individual identification and acclimated for 2 weeks to 12 ± 1°C and either a 8L:16D light-dark cycle (Experiment 2.1, February, 1980) or a 12L:12D light-dark cycle (Experiment 4.2, April, 1980), with lights on at 08:00 hr. In each experiment 8 groups of fish were maintained separately, in similar 96 ℓ flow-through aquaria. On the first day following the acclimation period blood samples were obtained from groups of fish at 3.43 hr intervals (i.e. 3 hr and 26 min) over a 24 hr period commencing at 24:00 hr. For convenience of presentation of the statistics in Figs. 4.1, 4.2 and 4.3, sample times were rounded to the hr nearest the midpoint of the sampling interval (i.e. 03:26 rounded to 03:00, 06:52 to 07:00, 10:18 to 10:00, 13:44 to 14:00, 17:10 to 17:00, and 20:36 to 21:00 hr). The method of anaesthesia, blood sampling procedure and preparation of the serum for RIA have been described previously (Cook and Peter, 1980). Each group was sampled over a 15 to 20 min interval and the maintenance of fish in individual hooded aquaria ensured that sampling of one group of fish did not influence the sampling of any other groups of fish. At 09:00 hr on the day following the first blood sample, the temperature was gradually raised to 20 ± 1°C. A second blood sample was obtained from each fish after 5 d at the elevated temperature, at the same time in the light-dark cycle as the first sample. During the acclimation periods the fish were fed Ewos (size 5P) trout chow (approximately 1% BWt/d) once per day, at preselected randomized times during the photophase. Feeding was withheld on the days of blood sampling.

Experiment 4.3

In this experiment, performed in August, 1980, all procedures including the acclimation time, method of feeding and blood sampling were similar to those described for Experiments 4.1 and 4.2 except that the fish were maintained under a 16L:8D light-dark cycle, and only a single blood sample was taken after an initial acclimation period to 20 ± 1°C. At the end of each experiment BWt, sex and gonad weight were noted for each fish. The gonosomatic index (GSI) for each fish was calculated as described by Cook and Peter (1980).

Experiments 4.4 and 4.5

The non-normal distribution and large variability of serum ir GH levels obtained in most of the groups sampled from the experiments outlined above suggested that serum ir GH levels might fluctuate in a pulsatile manner, similar to that described for mammals (see INTRODUCTION). For example, in Experiment 4.1, the serum ir GH levels ranged from 15.54 to 94.85 ng/ml in the group of fish held at 20 ± 1°C and sampled at 13:44 hr. Experiments 4.4 and 4.5 were designed to examine the cause of this variability in goldfish serum ir GH levels.

For Experiment 4.4, 126 male goldfish were acclimated for 4 weeks to a simulated ambient light-dark cycle (April, 1980) and 12 ± 1°C, and then randomly distributed into 2 groups of equal number in similar 296 ℓ flow-through aquaria. After acclimation for a further 10 d the tank temperature was gradually raised to 20 ± 1°C, since Experiments 4.1, 4.2 and 4.3 all suggested an increased occurrence of variable and elevated serum ir GH levels in fish acclimated to 20° compared to 12°C. After 5 d at 20°C, one group of fish was blood sampled at 09:00 hr and again 30 min later. Fish were fin-clipped for individual identification after the initial blood sample was obtained. The methods of anaesthesia, blood sampling and preparation of serum for the cGH RIA were as described for Experiments 4.1 to 4.3. The second group of fish were sampled in a similar manner at 16:15 and 16:45 hr.

For Experiment 4.5, 11 large female goldfish (104.80 ± 5.15 g BWt, $\overline{X}$ ± SE) were maintained and acclimated as described for Experiment 4.4. Blood samples (90 to 110 µl) were taken at 20 min intervals for a 2 to 3 hr period between 10:00 and 13:00 hr. In this experiment sampling was accomplished without anaesthesia using gentle restraint in foam rubber blocks. Serum was processed for the cGH RIA as described in Chapter 1.

III. Carp Growth Hormone Radioimmunoassay

The cGH RIA procedures and validation for the measurement of goldfish serum GH have been described in detail (see Chapter 1).

IV. Statistical Analyses

Experiments 4.1, 4.2 and 4.3 were analyzed by one-way analysis of variance and Duncan's multiple range test (Steel and Torrie, 1960) for differences between group means at each acclimation temperature. The data were normalized using a logarithmic transformation prior to statistical analysis. The paired Student's t-test (Steel and Torrie, 1960) was used to compare changes in serum GH levels between individual fish sampled at 12 ± 1°C and 20 ± 1°C, at each sampling time.

For Experiment 4.4 significant differences in serum ir GH levels between the first and second samples of individual goldfish were determined using the paired Student's t-test. The Student's t-test was used to compare mean serum ir GH values from the fish sampled at either 08:30 and 09:00 hr with those sampled at 16:15 and 16:45 hr. Since the sensitivity of the cGH RIA is approximately 5 ng/ml serum and since the precision of this assay permits detection of differences between separate samples of 5 ng/ml serum or less (see Chapter 1), changes in serum ir GH values of greater than 5 ng/ml between the 2 samples from individual fish were considered significant for the purposes of this experiment and for Experiment 4.5.

RESULTS

Experiment 4.1 (February, 8L:16D)

Fig. 4.1 shows the serum ir GH levels in female goldfish after acclimation to either 12 or 20°C. After acclimation to 12°C the mean serum ir GH level of fish sampled at 00:00 hr was significantly less than the groups sampled at 03:26, 10:18, 13:44 and 24:00 hr ($p < 0.01$). Serum ir GH levels measured at 06:52 and 20:36 hr were also significantly less than those obtained at 24:00 hr (Fig. 4.1). Although no significant daily fluctuations in serum ir GH were detected in the fish after acclimation to 20°C, there was a significant increase in serum ir GH over the levels found in the same fish acclimated to 12°C at 00:00, 06:52 and 20:36 hr. When all the samples taken at each temperature were pooled and compared to each other, it is evident that the mean serum ir GH levels are greater at 20° than at 12°C ($p < 0.001$) (Table 4.1). The GSI of the fish used in Experiment 4.1 was 4.11 ± 0.62 ($\overline{X}$ ± SE).

Experiment 4.2 (April, 12L:12D)

In Experiment 4.2 there were no significant differences in serum ir GH levels between groups of fish sampled at various times of day after acclimation to either 12° or 20°C (Fig. 4.2). The group of fish sampled at 10:18 hr had significantly elevated ir GH levels ($p < 0.01$) after acclimation to 20° compared to 12°C (Fig. 4.2); however, there were no significant differences between fish acclimated to 12° compared with those acclimated to 20°C when all the samples

Fig. 4.1. Serum immunoreactive growth hormone (ir GH) levels ($\overline{X}$ ± SE) from female goldfish, in February 1980, acclimated for 2 weeks to 12 ± 1°C and an 8L:16D light-dark cycle with lights on at 08:00 hr (——————) and after a further 5 d at 20 ± 1°C (--------------). The results of the Duncan's multiple range test ($p < 0.01$) are indicated; groups with common underscoring are not significantly different. Significant differences between the 12 and 20°C sample times were determined using the paired Student's t-test (*, $p < 0.05$). Note the $\log_{10}$ ordinate axis.

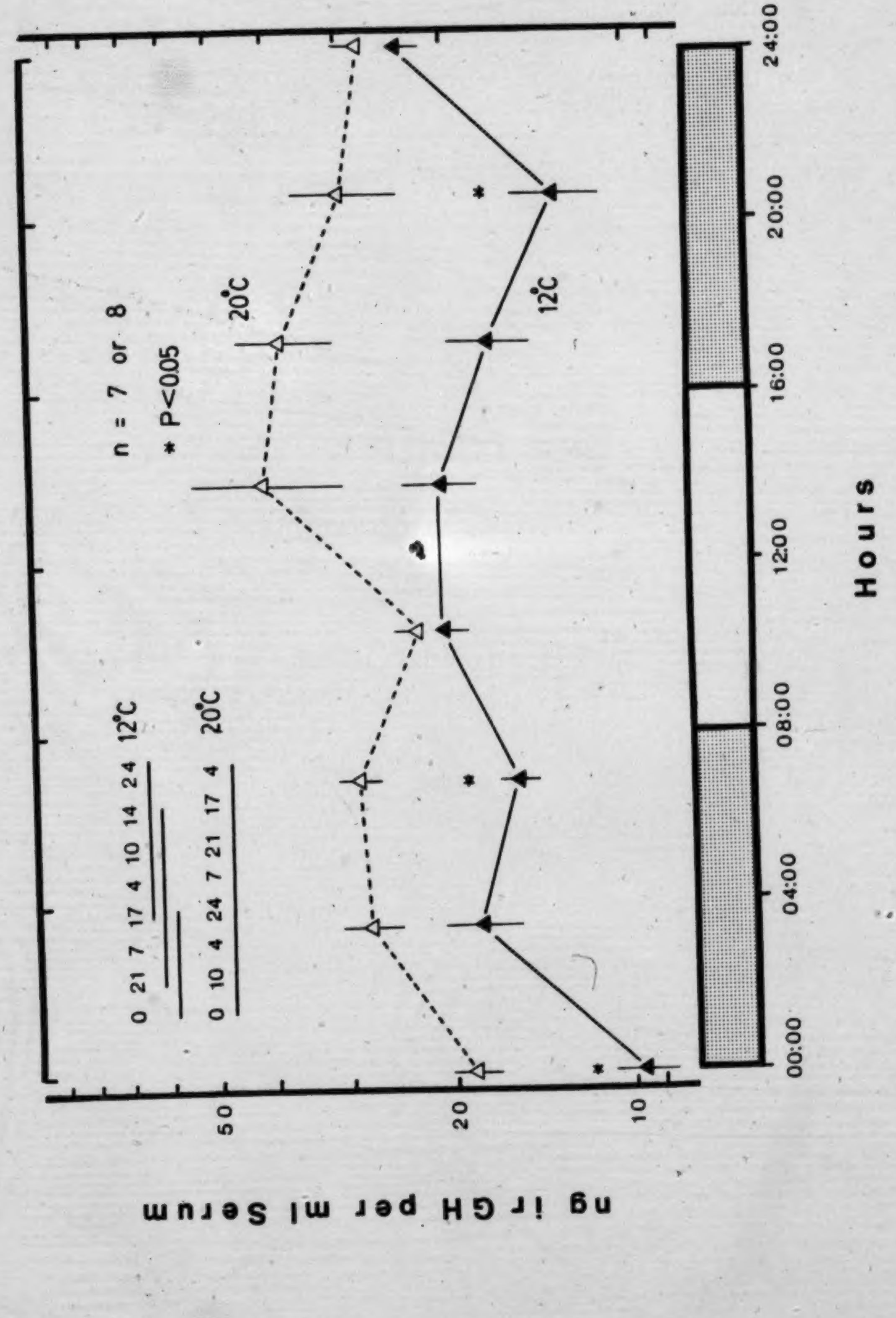

ng ir GH per ml Serum
10
20
50
Hours
00:00
04:00
08:00
12:00
16:00
20:00
24:00
n = 7 or 8
* P<0.05
20°C
12°C
0 21 7 17 4 10 14 24 12°C
0 10 4 24 7 21 17 4 20°C
*
*
*

TABLE 4.1

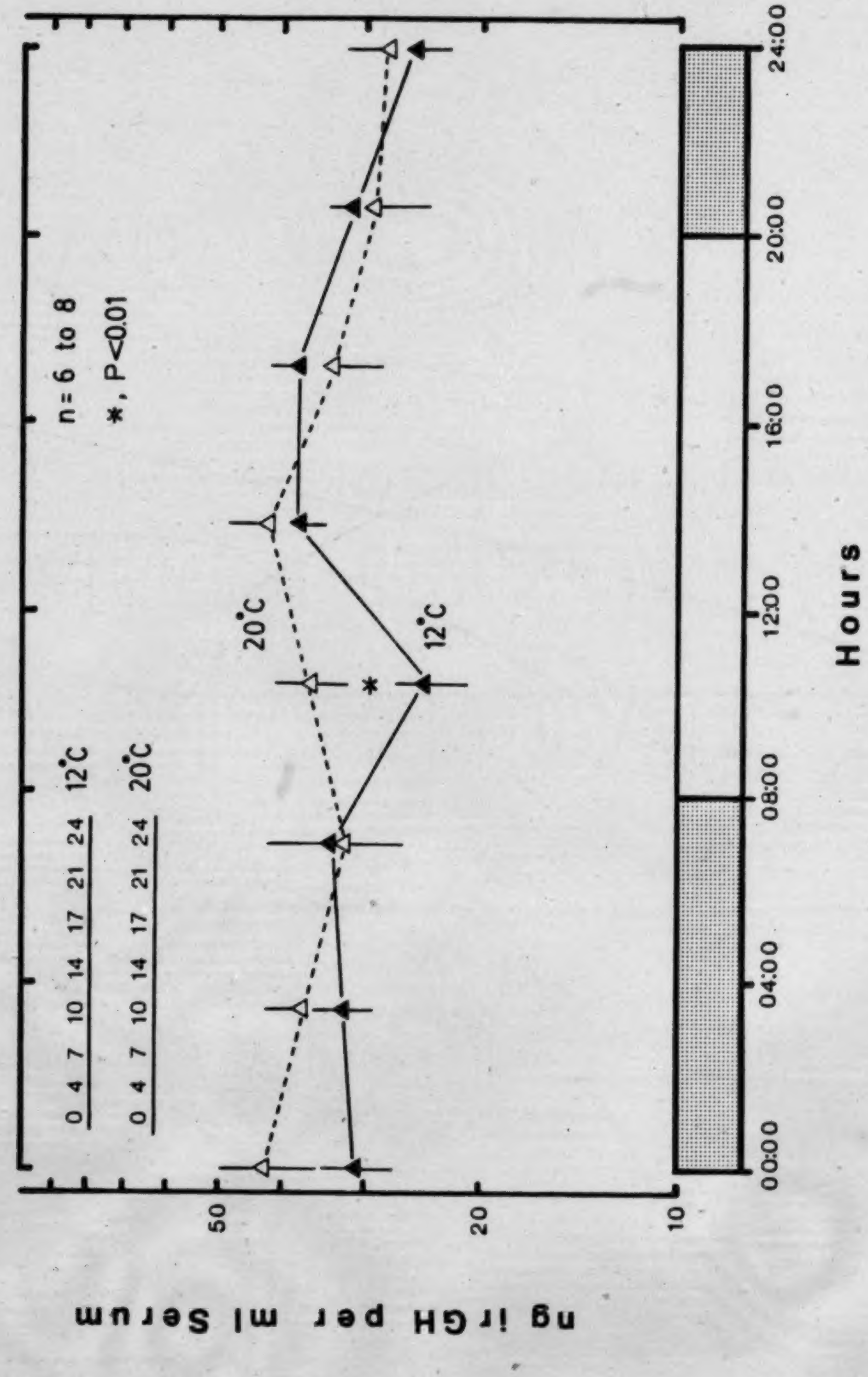
Average of serum immunoreactive growth hormone (ir GH) levels obtained at 8 separate times of the day in female goldfish held under different environmental regions and at different times of the year.

Experiment		4.1		4.2		4.3
°C		February		April		August
		8L:16D		12L:12D		16L:8D
		ng ir GH per ml serum				
	N	62		60		
12	$\bar{X}$	17.46[1]	($p < 0.001$)	31.89		
		±		±		
	SE	0.88		1.65		
		($p < 0.001$)[2]		(NS)		
	N	61		58		68
20	$\bar{X}$	29.23	($p < 0.05$)	35.91	($p < 0.001$)	55.88
		±		±		±
	SE	2.17		1.98		3.02

[1] Values are obtained by pooling the data from each of the 8 sample times of Experiments 4.1, 4.2 and 4.3.

[2] Significance levels determined using either the paired or unpaired Student's t-test, as appropriate (NS = nonsignificant).

Fig. 4.2. Serum immunoreactive growth hormone (ir GH) levels ($\bar{X}$ ± SE) from female goldfish, in April 1980, acclimated for 2 weeks to 12 ± 1°C and a 12L:12D light-dark cycle with lights on at 08:00 hr (————————), and after a further 5 d at 20 ± 1°C (--------------). The results of the Duncan's multiple range test ($p < 0.01$) are indicated; groups with a common underscoring are not significantly different. Significant differences between the 12 and 20°C sample times were determined using the paired Student's t-test (*, $p < 0.01$). Note the $\log_{10}$ ordinate axis.


ng irGH per ml Serum
50
20
10
00:00
04:00
08:00
12:00
16:00
20:00
24:00
Hours
0 4 7 10 14 17 21 24 12°C
0 4 7 10 14 17 21 24 20°C
n= 6 to 8
*, P<0.01
20°C
12°C
*

taken at each temperature were pooled for comparison (Table 4.1). The GSI of the fish used in Experiment 4.2 was 11.44 ± 1.01% ($\overline{X} \pm SE$).

Experiment 4.3 (August, 16L:8D)

Fig. 4.3 shows serum ir GH levels in female goldfish after acclimation to 20°C. The fish sampled at 10:18 hr had significantly lower ir GH levels compared with those sampled at 00:00 but not 24:00 hr. The GSI of the fish used in Experiment 4.3 was 1.37 ± 0.24%.

Table 4.1 presents a comparison of the serum ir GH levels in the female goldfish from Experiments 4.1, 4.2 and 4.3 after averaging of the values obtained at the 8 sample times of the 24 hr period. Fish sampled in April, after acclimation to 12° and 20°C (Experiment 4.2), had average serum ir GH levels that were significantly greater compared with those of the fish sampled in February (Experiment 4.1) after acclimation to the same temperatures. In turn, fish sampled in August (Experiment 4.3) had average serum ir GH levels which were significantly greater ($p < 0.001$) than those of fish sampled in April acclimated to 20°C (Table 4.1).

Experiment 4.4 and 4.5

The results of Experiment 4.4 are summarized in Table 4.2 and Fig. 4.4. The fish sampled at 16:15 hr had significantly greater serum ir GH levels than those sampled at either 09:00 or 09:30 hr (Table 4.2). Furthermore, serum ir GH levels in goldfish sampled at 16:45 hr were significantly elevated compared with those of the fish sampled at 09:30 hr. There was a significant decrease in serum ir GH

Fig. 4.3. Serum immunoreactive growth hormone (ir GH) levels ($\bar{X}$ ± SE) from female goldfish, in August 1980, acclimated for 2 weeks to 20 ± 1°C and a 16L:8D light-dark cycle, with lights on at 08:00 hr (--------------). The results of the Duncan's multiple range test ($p < 0.01$) are indicated; groups with common underscoring are not significantly different. Note the $\log_{10}$ ordinate axis.

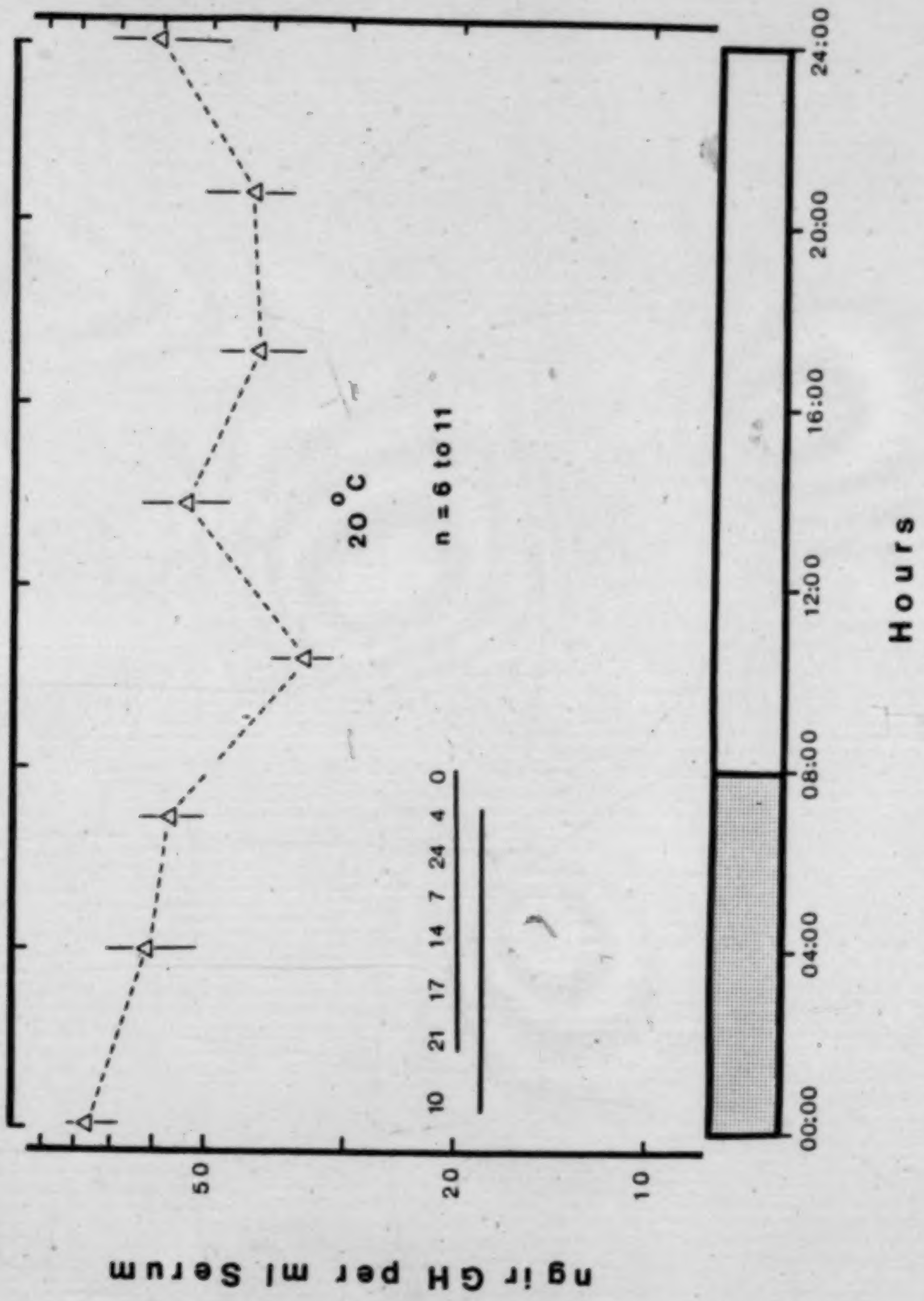

20 °C
n = 6 to 11
10 21 17 14 7 24 4 0
ng ir GH per ml Serum
50
20
10
00:00
04:00
08:00
12:00
16:00
20:00
24:00
Hours

TABLE 4.2

Serum immunoreactive growth hormone (ir GH) levels in two groups of male goldfish each sampled twice, 30 minutes apart in either the morning (Group A) or afternoon (Group B).

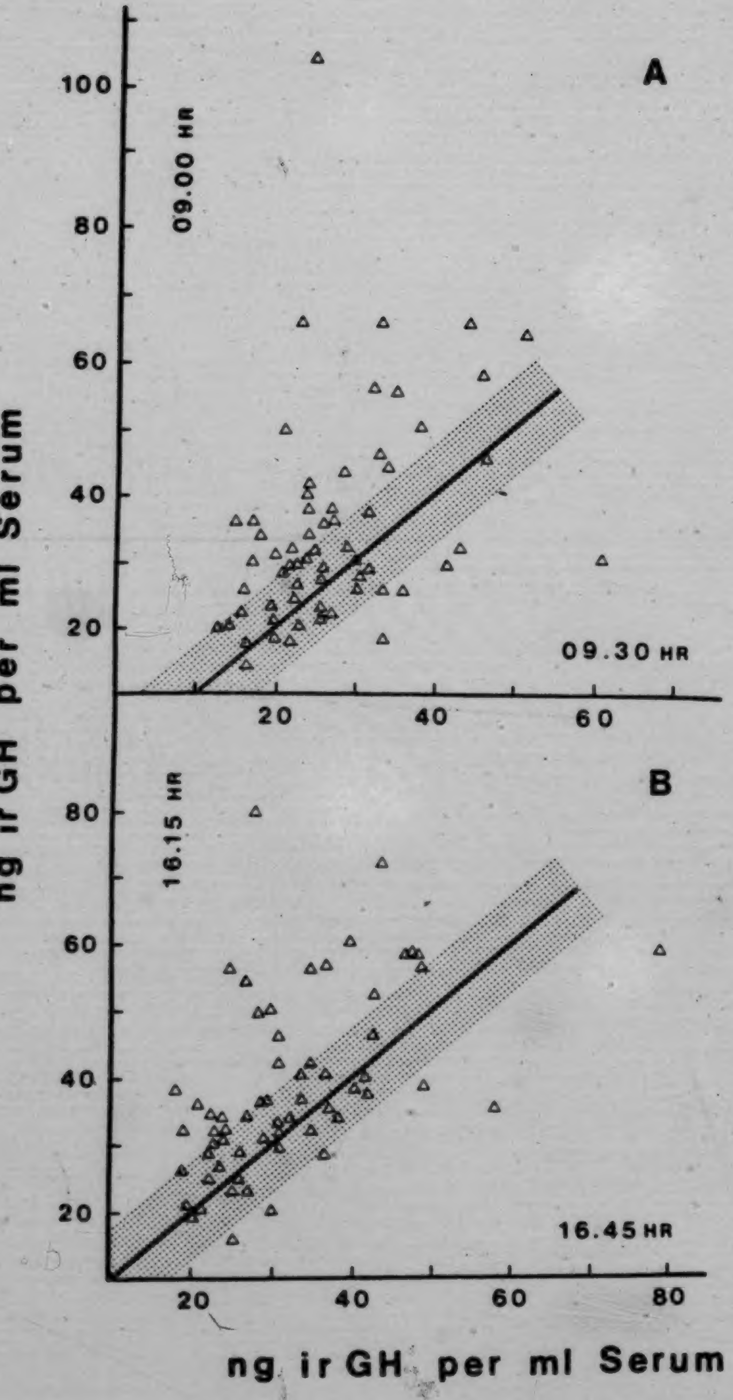
group	Group A		Group B
N	63		63
sample times (hr)	09:00, 09:30		16:15, 16:45
ng ir GH/ml serum	32.86[1] ± 2.00	($p < 0.01$)	38.22 ± 1.63
	($p < 0.01$)[2]		($p < 0.01$)
ng ir GH/ml serum	26.00 ± 1.25	($p < 0.001$)	32.48 ± 1.37

---

[1] All data are $\bar{X}$ ± SE.

[2] Significance levels determined using either the paired or unpaired Student's t-test, as appropriate.

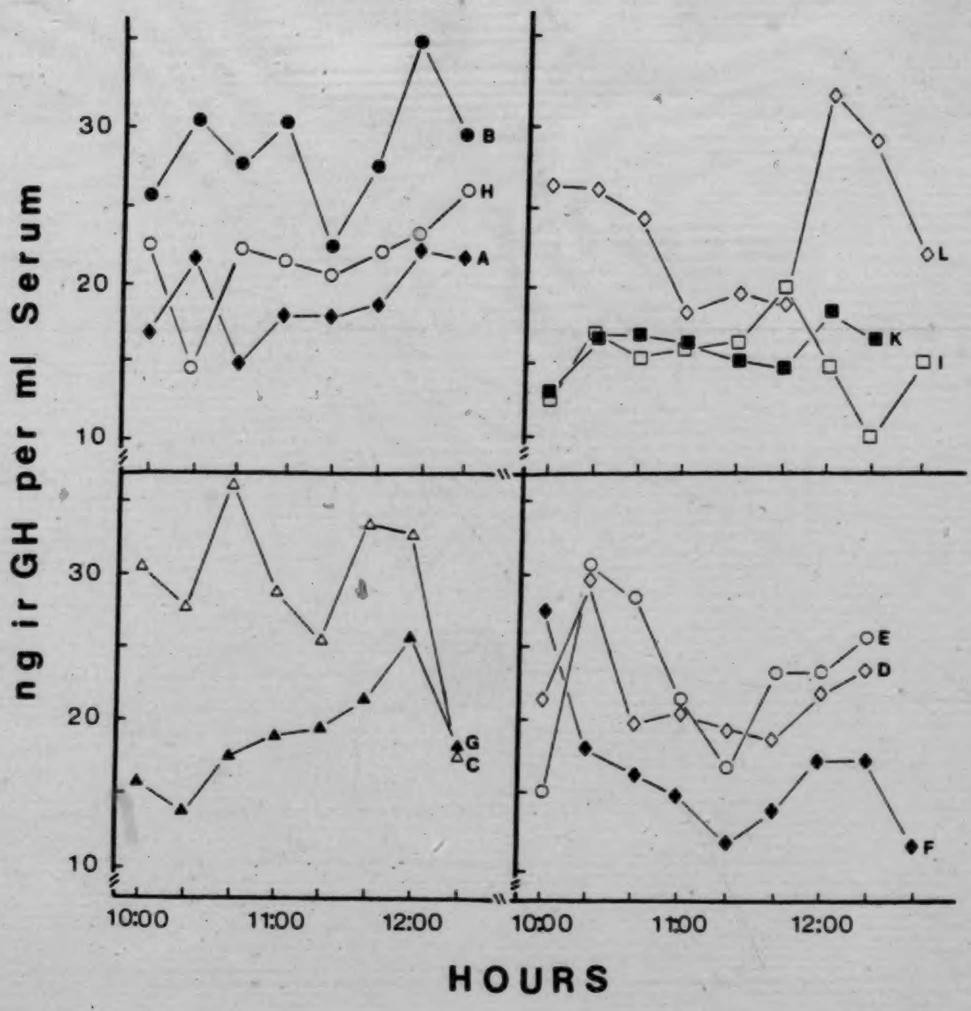
Fig. 4.4. Serum immunoreactive growth hormone (ir GH) levels in male goldfish acclimated to 20 ± 1°C. Each fish was blood sampled twice, 30 min apart in either the morning (A) or afternoon (B). The group sampled in the morning was sampled at 09:00 and 09:30 hr; the afternoon group was sampled at 16:15 and 16:45 hr. The figure illustrates the changes observed in individual fish in the serum ir GH levels at the first and second samples. The diagonal line and the shaded area represents a line of no change (i.e., slope = 1) and the shaded area around the line encompasses the fish which increased or decreased by less than 5 ng ir GH/ml serum. Five ng ir GH/ml serum is considered to be less than a detectable change because of assay sensitivity (see MATERIALS AND METHODS).


A
100
80
60
40
20
09.00 HR
09.30 HR
20
40
60
B
80
60
40
20
16.15 HR
16.45 HR
20
40
60
80
ng ir GH per ml Serum
ng ir GH per ml Serum

levels between the first and second samples in both groups of goldfish. Fig. 4.4 diagrams serum ir GH changes in individual fish sampled at either 09:00 and 09:30 hr (panel A) or 16:15 and 16:45 hr (panel B); the shaded area demarcates changes between the 2 sample times of less than 5 ng ir GH/ml serum. Analysis of the changes in serum ir GH levels in individual goldfish between the 2 blood samples taken at 09:00 and 09:30 hr (group A) indicated that about 90% of the fish in this group showed either significant (i.e. greater than 5 ng ir GH/ml serum difference between first and second sample; see MATERIALS AND METHODS) decreases (51%) or no significant change (38%); a small number of fish (10%) showed significant increases of between 5 and 31 ng ir GH/ml serum over the 30 min sampling interval (Fig. 4.4). Similarly, 52% of the fish sampled during the afternoon (group B) showed decreases of greater than 5 ng ir GH/ml serum between the 16:15 and 16:45 hr samples and 40% of the fish did not change by more than 5 ng ir GH/ml serum between these 2 sample times. As with the group of fish sampled at 09:00 and 09:30 hr, about 10% of the fish in group B showed increases greater than 5 ng ir GH/ml serum between the 16:15 and 16:45 hr samples. Fig. 4.4 also shows that the fish which show appreciable decreases during the experiment tend also to have elevated serum ir GH levels at the first sample (either 09:00 or 16:15 hr). There is no apparent relationship for fish which showed appreciable increases in serum ir GH levels.

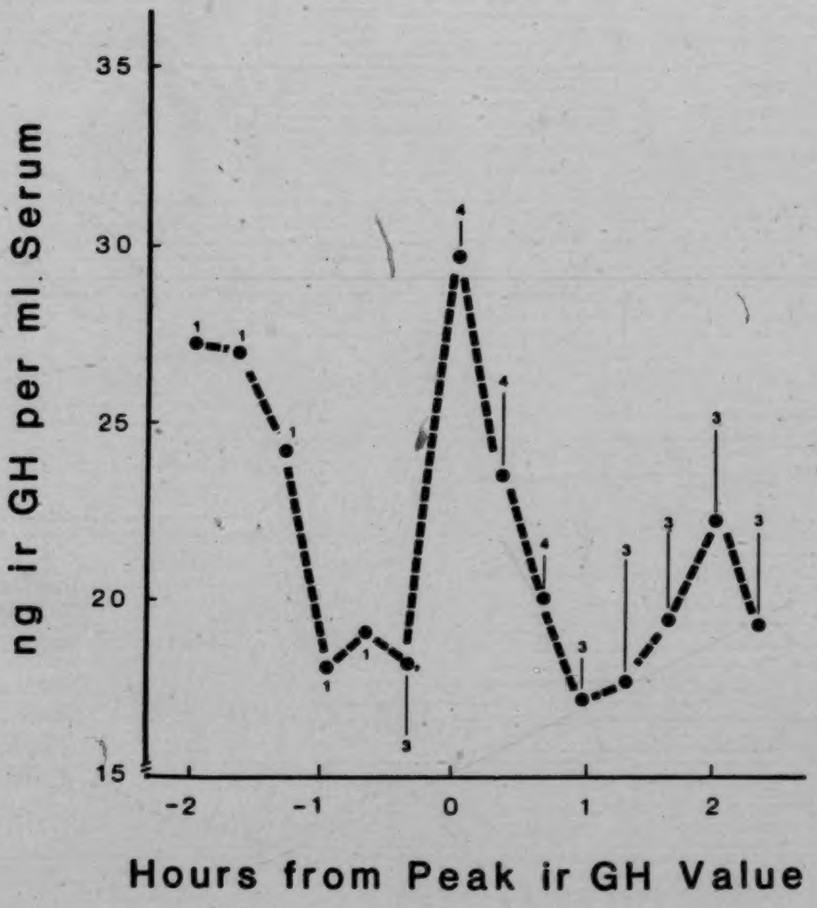
The results of Experiment 4.5 are shown in Fig. 4.5. Of the 11 fish sampled, all but 2 fish (I and K) showed changes of greater than 5 ng ir GH/ml serum within at least one 20 min interval of the

Fig. 4.5. Serum immunoreactive growth hormone (ir GH) levels obtained from large female goldfish serially sampled at 20 min intervals from 10:00 to 12:40 hr. The acclimation procedure, tank temperature and light-dark cycle were as described for Experiment 4.4. The series of samples from an individual fish are identified by a letter.


ng ir GH per ml Serum
30
20
10
30
20
10
B
H
A
L
K
I
G
C
E
D
F
10:00
11:00
12:00
10:00
11:00
12:00
HOURS

sampling period. Four fish (D, E, F and L) showed similar transient significant peaks followed by a slower decline to basal serum ir GH levels (Fig. 4.5). When the serum ir GH patterns for these 4 fish were shifted with respect to time in order to align the highest ir GH level, the composite pattern suggested that the increasing portion of the peak serum ir GH level occurred over a shorter time interval than the declining portion (20 min compared to 60 min; see Fig. 4.6).

Fig. 4.6. Serum immunoreactive growth hormone (ir GH) levels of serial blood samples obtained from fish D, E, F and L (same data as shown in Fig. 4.5). The sequence of values obtained at 20 min intervals from each fish was shifted with respect to those of the other fish to align the largest ir GH level (assigned to time 0 in the figure). The vertical bars represent the standard error and the number of values at each point are as shown.


ng ir GH per ml. Serum
35
30
25
20
15
1
1
1
1
1
3
4
4
4
3
3
3
3
3
-2
-1
0
1
2
Hours from Peak ir GH Value

DISCUSSION

Although circadian rhythms or significant daily fluctuations have been described for circulating levels of cortisol (Peter *et al.*, 1978), triiodothyronine and thyroxine (Spieler and Noeske, 1978), prolactin (McKeown and Peter, 1976) and gonadotropin (Hontela and Peter, 1978) in goldfish, the present study provided no evidence for reproducible daily variations in serum ir GH levels in this fish. Serum ir GH levels from groups of fish sampled at certain times were significantly different from other times during the same 24 hr period. For example, in Experiment 4.1 the groups of female goldfish acclimated to 12°C and sampled at 20:36 and 06:52 hr had significantly lower serum ir GH levels than those sampled at 24:00 hr. However, since individual serum ir GH levels were variable within each group of fish and since the 00:00 and 24:00 hr sample values were significantly different, it is not likely the observed patterns of serum ir GH levels in Experiment 4.1 are part of a circadian GH rhythm in the goldfish. In addition, there were no significant differences in serum ir GH levels at any of the sample times during the 24 hr sampling periods of Experiments 4.2 and 4.3 after acclimation to 12 and 20°C. However, these negative results regarding a possible circadian rhythm in serum GH in goldfish must be viewed cautiously since serum ir GH levels were significantly different when very large numbers of goldfish (N=63) were sampled at two separate times of day (Experiment 4.4). In addition, considering the results of Experiments 4.1 to 4.3, the possibility of a daily GH rhythm in goldfish kept under different environmental conditions can not be ruled out. However, since the pattern of ir GH levels obtained from serially sampling individual goldfish suggests that GH is secreted in a pulsatile manner (see below), there would have to be partial temporal synchrony of serum ir GH levels within a population of fish in order to detect a significant daily cycle of serum ir GH levels. Although only 11 fish were sampled repeatedly in Experiment 4.5, there was no evidence that the patterns of serum ir GH levels obtained from individual fish were synchronized with respect to time.

There is only limited published evidence available to support the concept of diurnal GH variations in teleosts. Leatherland *et al.* (1974) described a circadian rhythm in plasma GH levels in juvenile kokanee salmon, *Oncorhynchus nerka*, maintained at 10 to 12°C under a natural daylight photoperiod. Although these authors described significant GH peaks at 03:00 and 12:00 hr, they provided no data concerning reproducibility or stability of the 'rhythm' or whether similar patterns are present in salmon maintained under other conditions (Leatherland *et al.*, 1974). Furthermore, the heterologous GH RIA used in their study has not been sufficiently validated to permit an accurate assessment of the substance(s) measured (Nicoll, 1975; see GENERAL INTRODUCTION). More recently, Carillo *et al.* (1980) have shown a significant daily variation in nuclear area of presumptive GH cells in tilapia *(Sarotherodon mossambicus)* maintained at 22 to 23°C and either a 15L:9D or 9L:15D light-dark cycle. The finding that the nuclear area of GH cells was greatest at the end of the photophase and lowest at 8 hr after the end of the photophase in both short and long photophase experiments (Carillo *et al.*, 1980) suggested that this rhythm of the somatotrope cells is determined by either the start of the scotophase or end of the photophase. Without serum GH measurements it is not possible to make a direct comparison between theirs, and the present study, to determine if the species used, experimental conditions or other factors are responsible for the different results. Obviously, the question of possible circadian GH rhythms in teleosts remains open.

A number of investigators have assumed that GH secretion varies seasonally in fishes and can be influenced by environmental factors (e.g. Gross *et al.*, 1965; Swift and Pickford, 1965; Gerking, 1966; Saunders and Henderson, 1970; Komourdjian *et al.*, 1976; Adelman, 1977; Clarke *et al.*, 1981). The present study provides the first direct evidence for seasonal variations in serum GH levels in a teleost fish. Mean serum ir GH values (obtained by averaging all samples obtained during the single 24 hr sampling period) were significantly lower in fish sampled in February (8L:16D), after acclimation to 12°C, than in fish sampled in April (12L:12D) also acclimated to 12°C. Furthermore, in August (16L:8D) average serum ir GH levels of female goldfish acclimated to 20°C were significantly greater than those of fish sampled in February or April, after acclimation to 12 and 20°C. Since the serum ir GH values outlined above were obtained by averaging a large number (N=58 to 68) of individual serum ir GH measurements obtained from goldfish sampled at 7 equally spaced times of day, it is apparent that differences in results of Experiments 4.1 to 4.3 are more likely related to either the time of year or photoperiod rather than the temporal pattern of GH release in individual fish (see below) or time of day of blood sampling.

The finding that goldfish serum ir GH levels, averaged over a 24 hr period, were significantly elevated in August compared to those of either February or April, suggests that the increased growth rates observed in the post-spawning period, usually during the late spring and early summer months for a variety of fishes (see INTRODUCTION), is related to an increase in GH secretion. It is important to emphasize, however, that the experimental design of the present study does not permit definitive conclusions regarding the cause of the difference in average serum ir GH levels between Experiments 4.1 to 4.3. Since both time of year and the experimental light-dark cycle changed concurrently, either or both of these factors may have contributed to the observed results. Although the experimental protocol and blood sampling procedure used in the present study prevented the simlutaneous determination of growth rates and serum ir GH levels, other studies in this laboratory have shown that growth in goldfish, acclimated during August to 20°C and a 16L:8D light-dark cycle, is significantly faster than that of fish maintained at 20°C and a 12L:12D light-dark cycle during April (A.F. Cook, unpublished results).

Studies concerning the periovulatory changes in circulating hormone levels from a natural population of white suckers, *Catastomus commersoni*, have indicated significant differences in serum ir GH levels at the time of spawning (N.E. Stacey, A.F. Cook, D. MacKenzie and R.E. Peter, unpublished results). Ir GH measurements obtained from female suckers prior to and during ovulation (35 and 47 ng ir GH/ml serum, N=19 and 11, respectively) were significantly less than those from spent fish (74 ng ir GH/ml serum, N=9). Since spent (i.e. post-spawning) white suckers are known to leave the spawning grounds within several days following ovulation (Scott and Crossman,1973; N.E. Stacey, personal communication) and since serum ir GH measurements from spent fish sampled at the spawning grounds are greater than those from ovulating fish, it is likely that GH secretion increases soon after spawning in this species. It is not known if serum ir GH levels are elevated as quickly after ovulation in the goldfish as in the sucker, but the present study has shown that serum ir GH levels of sexually regressed female goldfish in summer (August) are greater than in pre-ovulatory females sampled in the spring (April). In addition, serum ir GH measurements from sexually regressed goldfish (GSI = 1.37 ± 0.24%, $\overline{X}$ ± SE) sampled in August (Experiment 4.3, 55.88 ± 3.02 ng ir GH/ml serum) were greater than ir GH levels obtained from 2 preovulatory goldfish (GSI = 9.08 ± 2.00%; 29.28 and 44.94 ng ir GH/ml serum) held under identical conditions at the same time of year.

Since increasing ambient temperature from 12 to 20°C resulted in a marked increase in serum ir GH levels in February (8L:16D), but not in April (12L:12D), it is apparent that, in addition to reproductive state, the season (i.e. month of year) and/or light-dark cycle can modify the GH response to temperature. A large number of studies have demonstrated that, in fishes, the growth response increases with elevated temperature up to an optimum, then declines at above optimal temperatures (for review: Brett, 1979). Since it has been shown that elevated serum ir GH levels can be associated with elevated growth rates in goldfish (see Chapter 3), the serum ir GH response to a temperature increase observed in February, but not April, may represent part of a pituitary mediated temperature effect on growth of goldfish. Together, the effects of temperature and season and/or photoperiod on serum ir GH levels in goldfish make it possible to speculate that GH secretion increases progressively during the period from early spring to the summer months, with maximum levels associated with the period of most rapid somatic growth occurring after the spawning season and that GH secretion is accelerated by increasing ambient temperature in the early spring. The present study has provided support for the presumed relationship between seasonal growth and GH secretion in teleosts (see INTRODUCTION) and has demonstrated that the goldfish, and the cGH RIA described previously, will be useful to further our understanding of the role of various environmental factors in influencing circulating GH levels in fishes.

Serum ir GH levels in goldfish from a variety of experiments were often non-normally distributed with low values bunched close to the mean and elevated values extending far above the mean. In Experiment 4.4 the distribution of serum ir GH values from the group of goldfish sampled at 09:00 hr was significantly different from a normal distribution of values ($p < 0.05$, Kolmogorov-Smirnov test of normality, $D_{max} = 0.178$), and a logarithmic transformation of the values resulted in a normal distribution. Since there was a significant difference between the serum ir GH values of individual fish sampled at 09:00 and 09:30 hr it is likely that the non-normal distribution is a result of the changing serum ir GH levels in individual fish, rather than a non-normal distribution of constant serum ir GH in individual goldfish. The finding that serum ir GH levels can change markedly in the 30 min sampling interval of Experiment 4.4 suggests that GH may be released in spontaneous bursts similar to that described for mammals (for review: Martin, 1976; Weiner and Ganong, 1978; see INTRODUCTION). If it is assumed there is a non-uniform distribution of GH secretory bursts with respect to time, as suggested by Experiment 4.5, and that the effect of sampling on serum ir GH levels is similar for all goldfish, then the finding that more fish showed decrements than showed increments over the 30 min period, suggests that the time-interval of a 'GH secretory burst' is much shorter than that of the subsequent decline to basal ir GH levels. Although it is possible that the stress of the first blood sample may have influenced the second serum ir GH measurement, the finding that both the magnitude and direction of change between the two samples was different in individual goldfish, in spite of their similar BWt, suggests that the observed results were not due to blood sampling. In addition, data obtained by serially sampling large goldfish also demonstrated the pulsatile variations in serum ir GH levels and confirmed that the bursts occur over a shorter time period (about 20 min) than the declining period (about 1 hr) (see Fig. 4.6).

The short-term fluctuations in serum ir GH levels shown in Figs. 4.4 to 4.6 were of a smaller amplitude than that observed in other studies in goldfish (A.F. Cook, unpublished results) and in the rat and other mammals (for review: Chiodini and Liuzzi, 1979).

While it is possible that the secretory GH patterns are smaller in goldfish than in mammals, additional studies are required to determine whether the method of anaesthesia or blood sampling procedure are, in part, responsible for this difference. In addition, future studies will have to determine the effect of feeding on pulsatile GH release in goldfish in view of recent studies in the rat where food deprivation for 48 and 72 hr was shown to suppress pulsatile GH release (Tannenbaum *et al.*, 1979; Tannenbaum, 1981).

While additional studies are required to determine if the changing serum ir GH levels are part of an ultradian rhythm or represent random GH secretory bursts, the present results strongly suggest pulsatile GH release in goldfish. Since the present study has also demonstrated seasonal variations in goldfish serum ir GH levels that can be related to the seasonal pattern of growth typical of many spring-spawning teleost fishes, it is possible that the seasonal variations in GH secretion in goldfish represent superimposed neuroendocrine influences on the pulsatile secretory mechanism.

GENERAL DISCUSSION

The major objective of the present study was the development of a valid RIA suitable for the measurement of circulating GH levels in goldfish. Results presented in Chapter 1 demonstrated that the cGH RIA provided reproducible and sensitive measurements of ir GH in goldfish serum. The sensitivity and range of this RIA was suitable for the wide variety of goldfish physiological studies described in Chapters 2 to 4.

The specificity of the cGH RIA for endogenous circulating GH in goldfish was investigated using several independent procedures which are summarized below. First it was demonstrated that serial dilutions of serum from goldfish with an intact pituitary gland caused RIA inhibition curves which were parallel to that of purified cGH, whereas serum from hypox goldfish did not cross-react in this RIA. Second, goldfish serum ir GH levels were not altered under conditions which were associated with marked changes in GTH and PRL secretion. Third, the ir GH measurements of goldfish serum which had been fractionated by gel filtration (Sephadex G-100) indicated chromatographic similarity between serum ir GH and purified ^{125}I-cGH. Fourth, immunoadsorption of goldfish serum with rabbit anti-cGH serum and subsequent gel filtration suggested that the rabbit anti-cGH serum used in the RIA binds to circulating goldfish GH, supporting the specificity of the cGH RIA. Fifth, analysis of RIA inhibition curves caused by serial dilutions of carp GTH and goldfish PRL indicated that the contribution of these hormones to serum ir GH measurements is either negligible or nonexistent. Sixth, results obtained using the PAP immunohistochemical technique extended the RIA analyses of hormone specificity by demonstrating that the rabbit anti-cGH serum reacted only with the electron dense granules of the growth hormone cells in the goldfish pituitary gland. Since the presumptive hormone granules of the other goldfish pituitary cell types did not react using the PAP method and the rabbit anti-cGH serum, it is likely that only goldfish GH reacts in the RIA.

The biological activity of the cGH used for standards and tracer in the RIA was determined by measuring its growth promoting activity in a near-homologous bioassay. Intact goldfish injected with 1 µg cGH/g BWt gained weight at a significantly greater rate than either the pretreatment control values or simultaneous vehicle injected groups. Preliminary results obtained by passive immunization of goldfish with rabbit anti-cGH serum suggested that endogenous circulating goldfish GH was neutralized by this antiserum. Although further investigations are required to fully evaluate the biological activities of both the cGH and the rabbit anti-cGH serum, the present study has demonstrated that the cGH RIA is based on a growth-promoting hormone and growth-neutralizing antisera.

A series of experiments described in Chapter 2 demonstrated an inhibitory action of synthetic mammalian SRIF on serum ir GH levels in male goldfish. Two ip injections of either 0.5 or 1.0 µg SRIF/g BWt given 12 hr apart caused a significant decrease in serum ir GH levels at 1.5 hr following the second injection and also resulted in a significant post-inhibitory rebound in serum ir GH levels at 24 hr post-injection. Analysis of the changes in serum ir GH levels in individual goldfish suggested that SRIF may act by inhibiting only the spontaneous GH secretory bursts (see below). Since SRIF also inhibits GH release in a variety of mammals and in birds (Chiodini and Liuzzi, 1979), the cGH RIA developed in the present study shares, in common with a wide variety of independently developed GH RIAs, the property of measuring the inhibition of serum ir GH levels after SRIF treatment. In addition, these results extend previous studies by Fryer *et al.* (1979) who demonstrated that SRIF produced a dose-dependent inhibition of GH release from tilapia pituitary glands cultured *in vitro*. Since SRIF has been shown to inhibit GH release *in vitro* (tilapia GH measured by RRA, Fryer *et al.*, 1979) and *in vivo* (goldfish GH measured by RIA, present study), and has been located in several hypothalamic nuclei, especially the NPP (rainbow trout, SRIF detected by immunofluorescence, Dubois *et al.*, 1979), in teleost fishes, it is now reasonable to postulate a role for SRIF in the regulation of GH secretion in this vertebrate group. Further support for this hypothesis is provided by results of brain lesioning studies which indicated the NPP of the goldfish hypothalamus was involved in the inhibition of GH secretion in this species (Fryer, 1981; see Chapter 3).

Additional experiments presented in Chapter 2 investigating the effects of a variety of monoamines and related drugs and their interaction with SRIF on serum ir GH levels in goldfish also provided new information regarding the neural regulation of teleost GH secretion.

Systemic injections of 50 to 100 μg L-DOPA/g BWt resulted in a dose-dependent elevation in serum ir GH levels at 1 hr post-injection. The finding that the serum ir GH response to a combination of L-DOPA and the peripheral decarboxylase inhibitor CARBIDOPA was greater than that of an equivalent dose of L-DOPA alone suggests a CNS site of action for L-DOPA in elevating serum ir GH levels in goldfish. Since NE decreased serum ir GH levels during the summer months at a time when L-DOPA increases serum ir GH levels, it is likely that DA, and not NE, is responsible for the central stimulatory effect of L-DOPA on GH secretion in goldfish. Further work is necessary to determine whether this action of DA on serum ir GH levels is mediated by the action of SRIF and/or GHRF on the somatotrope secretory cells of the goldfish pituitary gland.

Results of experiments completed between November and February provided evidence for an α-adrenergic stimulation of GH secretion in the goldfish, whereas similar experiments done in the summer months demonstrate that NE lowered serum ir GH levels. The finding that the serum ir GH response to exogenously administered NE varied depending on time of year makes it reasonable to consider the possibility of a central GH regulating mechanism in goldfish that may be influenced by the season and/or reproductive state (see DISCUSSION of Chapter 4). In support of this, results of hypothalamic lesioning in goldfish also differed when experiments were done at different times of year (see Chapter 3) and serum ir GH levels were also found to vary seasonally in goldfish (see Chapter 4). Further investigations concerning the control of GH secretion in fishes must take into account the possible influence of time of year and environmental factors on experimental results.

Lesions placed in the NPP of the goldfish hypothalamus resulted in significant increases in both BWt and SL increments, and serum ir GH levels at 4 weeks post-operation. In rainbow trout, the NPP has been shown to be rich in SRIF immunoreactivity, presumably reflecting the high concentration of somatostatinergic neurons in this hypothalamic nucleus (Dubois *et al.*, 1979). If the distribution of SRIF in the goldfish hypothalamus is similar to that of the rainbow trout, then the increased serum ir GH levels and histological and ultrastructural evidence of increased GH secretion (Fryer, 1981) following NPP lesioning in goldfish was likely due to destruction of somatostatinergic neurons and subsequent removal of the inhibitory influence of SRIF on GH secretion. Since changes in growth, as indicated by increased BWt and SL increments, and serum ir GH levels were both elevated after lesioning of the NPP in goldfish, it is reasonable to speculate that increased growth rates resulted from increased pituitary GH secretion. In addition, these findings are consistent with the hypothesis that the cGH RIA measures circulating ir GH in goldfish that is, at least in part, biologically active.

The cGH RIA described above was used in Chapter 4 to determine if goldfish serum ir GH levels vary during either a daily or hourly time period. Since the daily sampling experiments (i.e. 8 groups of fish sampled during a 24 hr interval) were done at three separate times of year, it was also possible to determine whether serum ir GH levels in female goldfish vary depending on either season and/or photoperiod. Results presented in Chapter 4, based on three separate experiments (4.1, 4.2 and 4.3), provided no evidence for a circadian rhythm in serum ir GH levels in goldfish. Although the present study has clearly demonstrated abrupt changes in serum ir GH levels in goldfish (see below) suggestive of pulsatile GH release, it is premature to speculate on the possible presence of an ultradian GH rhythm in teleosts until future studies have carefully examined the influence of the blood sampling procedure and time of feeding on the pattern of GH release in fishes. The finding that serum ir GH levels in individual goldfish can fluctuate abruptly over a short time interval of about 20 to 30 min and the apparent lack of synchrony in serum ir GH changes between fish are both consistent with the lack of a circadian rhythm in serum GH.

An important finding presented in Chapter 4 was the marked differences in serum ir GH levels in female goldfish sampled at different times of year. Mean serum ir GH values (obtained by averaging all samples obtained during the single 24 hr sampling period) were significantly lower in February (8L:16D), after acclimation to 12°C, than in fish acclimated to 12°C in April (12L:12D). In addition, there were progressive increases in mean serum ir GH levels in fish sampled in February (8L:16D), April (12L:12D) and August (16L:8D), all acclimated to 20°C. Since growth rates of many spring-spawning teleost fishes are greater in the late spring and summer months after the spawning period, it is reasonable to speculate that the increased serum ir GH levels observed in the female goldfish sampled in August reflect increased pituitary GH secretion and subsequent growth-promoting action of circulating GH.

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APPENDIX I

FRACTIONATION OF CARP GROWTH HORMONE AND PREPARATION OF ANTISERA CARRIED OUT AT THE HORMONE RESEARCH LABORATORY, UNIVERSITY OF CALIFORNIA, SAN FRANCISCO, BY DR. SUSAN WALKER FARMER.

MATERIALS AND METHODS

Acetone dried carp pituitary powder was purchased from Stoller Fisheries, Spirit Lake, Iowa, in two batches of 21 g and 8 g dry weight. The methodology employed for the purification of GH from these pituitaries was similar to that previously employed for other GHs (Farmer *et al.*, 1976, 1981). Methodological details presented in these reports will not be repeated here. The pituitary powder was dissolved in water, adjusted to pH 9.5 with $Ca(OH)_2$ and stirred at 4^o for 3 hr. The soluble extract was chromatographed on Amberlite cG-50 equilibrated with $(NH_4)_2SO_4$, and the GH fraction was eluted with pH 6 phosphate buffer. This fraction was then chromatographed on DEAE-cellulose equilibrated with 0.03 M NH_4HCO_2, pH 9; GH was adsorbed and eluted with 0.2 M NH_4 HCO_3. After removal of a 20% precipitate the GH fraction was precipitated with 40% $(NH_4)SO_4$, and then with HPO_3, in order to further concentrate the GH. Final purification was achieved by gel filtration on Sephadex G-100 equilibrated with 0.05 M NH_4HCO_3.

As noted above, two batches of carp pituitary powder were processed. A low yield of GH was obtained with the initial batch, 5 mg/kilo, which may be due to the fact that improperly chilled acetone was used for the gland collection. A higher yield, 75 mg/kilo, was obtained with the second batch. This material was used for characterization and radioiodination while GH from the first batch was used for antiserum production.

The following determinations were performed on the cGH preparation: amino acid composition (Spackman *et al.*, 1958); NH_2-terminal amino acid analysis by the Dansyl procedure (Gray, 1967; Woods and Wang, 1967); COOH-terminal analysis by hydrazinolysis (Niu and Fraenkel-Conrat, 1955); molecular weight determination by SDS polyacrylamide disc gel electrophoresis (Weber and Osborn, 1969); and gel electrophoresis at pH 8.3 in 7.5% gels stained with Coomassie blue dye (Ornstein, 1964). Double antibody RIAs employed for identification of GH fractions were based on a monkey anti-rat GH serum (Hayashida, 1970) and a monkey anti-snapping turtle GH serum (Hayashida *et al.*, 1975), with iodinated rat GH (National Institute of Arthritis and Metabolic Diseases).

For preparation of antiserum to the purified carp growth hormone, a young male albino New Zealand rabbit was injected with cGH prepared from the first batch of pituitaries (see above). Three injections of 300, 250 and 200 μg each were given in complete Freund's adjuvant at 20 to 30 d intervals. A booster injection of 50 μg was given in saline and the rabbit was completely bled by cardiac puncture. Merthiolate (1:10,000) was added to the serum as a preservative.

RESULTS

Carp growth hormone behaved identically during purification to the tetrapod and piscine GHs previously studied (Farmer *et al.*, 1974, 1976, 1981). cGH was obtained in a low yield, 3 mg total, representing 75 mg/kilo, precluding detailed characterization studies. A molecular weight of 22,500 was calculated for cGH. Leucine was found as the major amino terminal residue, but trace amounts of several other amino acids were also identified. Serine was the major carboxyl terminal residue, glycine was also present. On disc gel electrophoresis, cGH showed the same pattern as the other GHs, but had a slightly higher R_f value (about 0.35), similar to human GH (Fig. I.1). The amino acid composition of cGH is present in Table I.1, along with that of two other piscine species, tilapia and sturgeon. Because the molecular weights of these GHs are all similar to that of ovine GH, the piscine amino acid analyses were calculated on the basis of the number of residues determined by structural analysis for ovine GH (191 residues; Li *et al.*, 1973). The cGH exhibits features that have been found to characterize known vertebrate GHs; two disulfides, a single tryptophan, low methionine and histindine content, and a high glutamic acid and leucine content (Wilhelmi, 1974). The cGH composition had high values for aspartic acid and valine content, but all values were within observed ranges of GH molecules from a wide variety of species (for reviews: Wilhelmi, 1974 for mammalian species and Farmer and Papkoff, 1979 for non-mammalian species).

Testing of cGH and tGH for immunological reactivity in two heterologous GH RIA systems which show wide species cross-reactivity, demonstrated significant, low-potency slopes of inhibition compared to that of the rat GH standard (Fig. I.2). Notably the cGH and tGH showed displacement curves which were approximately parallel compared to each other in both of these GH RIAs (see Fig. I.2).

Fig. I.1. Disc gel electrophoresis patterns of purified mammalian and piscine pituitary growth hormones. The gels were 7 ½% polyacrylamides, run at pH 8.3, and stained with Coomassie blue dye.

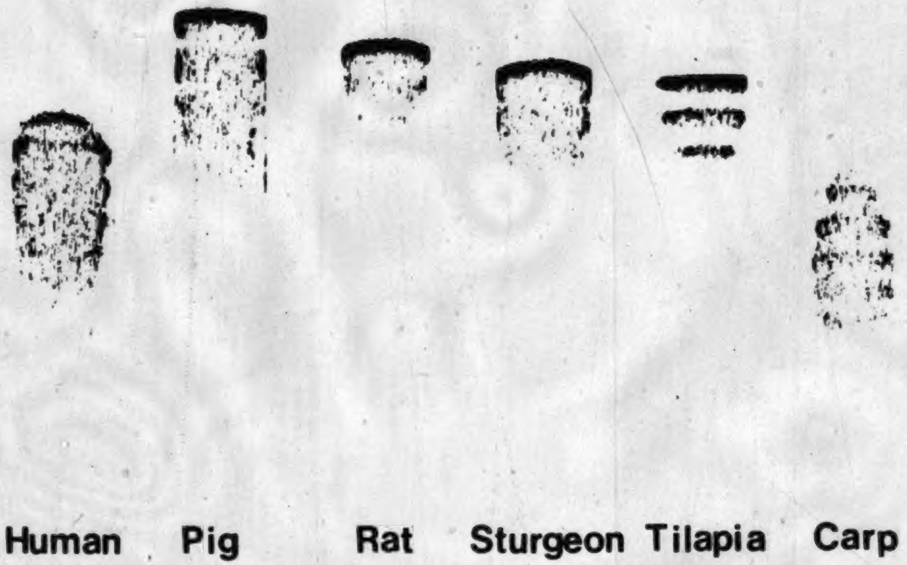

Human
Pig
Rat
Sturgeon
Tilapia
Carp

TABLE I.1

Amino acid composition[1] of carp, tilapia[2] and sturgeon[3] growth hormones.

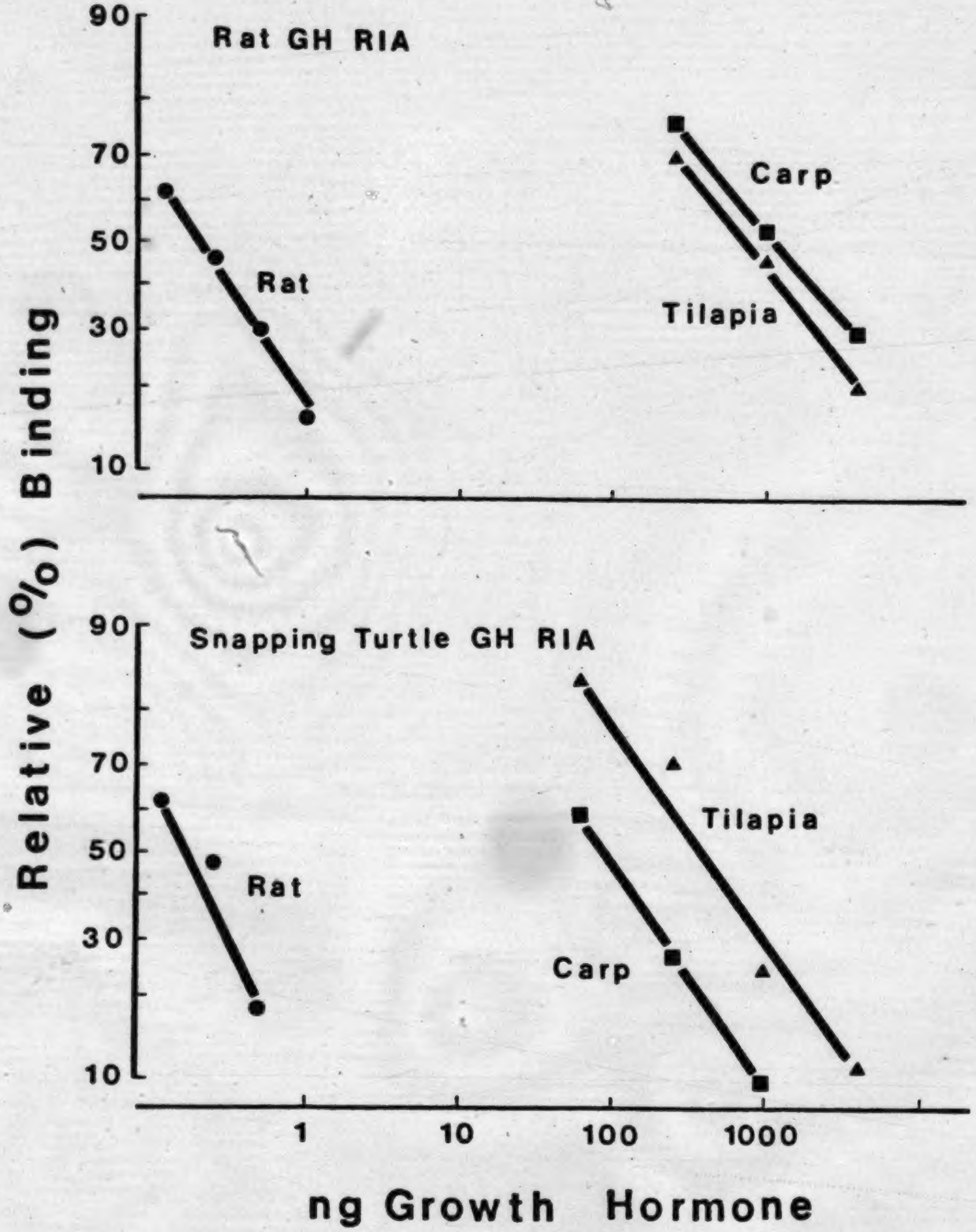
Amino acid	Carp	Tilapia	Sturgeon
Lys	10.4	8.2	15.2
His	4.8	5.0	4.0
Arg	9.3	11.0	11.0
Asp	24.8	19.3	20.3
Thr	10.1	12.0	9.7
Ser	16.5	21.4	18.3
Glu	23.6	29.1	21.9
Pro	8.9	6.8	6.7
Gly	8.8	7.4	7.6
Ala	10.6	8.2	9.3
½ Cys	3.7	4.6	4.6
Val	11.8	6.0	9.3
Met	4.5	1.2	4.9
Ile	7.0	9.0	5.5
Leu	22.8	27.2	24.1
Tyr	4.5	7.2	6.3
Phe	8.0	6.7	10.5

[1] Amino acid analysis: 20 hr hydrolysis, not corrected for hydrolytic destruction, calculated on the basis of 191 residues/mole.

[2] taken from Farmer *et al.* 1976.

[3] taken from Farmer *et al.* 1981.

Fig. I.2. Competitive binding curves for preparations for purified rat, tilapia and carp growth hormones (GH) in two double antibody radioimmunoassays (RIA) employing a rat GH antiserum and a snapping turtle GH antiserum. Rat GH was employed as a tracer for both as assays. Each point represents the mean of duplicate determinations.


Rat GH RIA
Carp
Rat
Tilapia
Snapping Turtle GH RIA
Tilapia
Rat
Carp
90
70
50
30
10
1
10
100
1000
Relative (%) Binding
ng Growth Hormone